University of Alberta

Short and Long Term Regulation of Ion Channels by Neurotransmitters

by

Christopher P. Ford

A thesis submitted to the Faculty of Graduate Studies and Research in
partial fulfillment of the
requirements for the degree of Doctor of Philosophy

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Abstract

This thesis examined both the acute and the chronic regulation of ion channels in fully developed adult bullfrog sympathetic ganglia (BFSG) neurons by neurotransmitters acting via G-protein coupled receptors.

A variety of neurotransmitters excite neurons by suppressing a ubiquitous, voltage-dependent, non-inactivating K^+ conductance called the M-conductance (g_M), however the mechanism responsible remains unknown. The results presented in this thesis are consistent with the involvement of the lipid and inositol phosphate cycles in the effects of P2Y, muscarinic cholinergic, and luteinizing hormone releasing hormone (LHRH)-receptor agonists on g_M. Suppression of g_M by all three agonists involved phospholipase C (PLC), but not 'downstream' products of PLC, such as inositol trisphosphate (IP_3), Ca^{2+}, or protien kinase C (PKC). Impairment of re-synthesis of phosphatidylinositol 4,5, bisphosphate (PIP_2) slowed or blocked recovery of agonist-induced g_M suppression. Neutralizing PIP_2-M-channel interactions inhibited steady state g_M and protecting PIP_2 from hydrolysis by PLC reduced LHRH-induced suppression. Intracellular application of PIP_2 slowed the run-down of KCNQ2/3 currents expressed in COS-1 or tsA-201 cells and PIP_2 produced a small potentiation of native M-currents. These are the results which might be expected if agonist-induced activation of PLC and the concomitant depletion of PIP_2 contributes to the excitatory action of neurotransmitters that suppress g_M.

This thesis also exploited the simple organization of BFSG to test whether LHRH, the neurotransmitter for the late slow e.p.s.p, could also exert long-term neurotrophic control of ion channel expression. Removing all *in vivo* sources of ganglionic LHRH for

10 days by cutting preganglionic C-fibers reduced Ca^{2+} current density. Dissociated BFSG B-neurons exhibited an increased I_{Ca} density after 6-8 days in the presence of LHRH. This increase was not associated with alterations in activation or inactivation kinetics of the Ca^{2+} channel conductance, and reflected an increase in N-type Ca^{2+} channel current. The increase in I_{Ca} density induced by LHRH was blocked by a transcription inhibitor, and inhibitors of Ras isoprenylation, MAPK-kinase, protein kinase A, protein kinase C and phospholipase C. These results suggest that chronic activation of G-protein coupled receptors in a fully-differentiated, adult sympathetic neuron *in vitro* or *in vivo* exerts long term-control of ion channel expression.

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Table of Contents

Chapter 1

General Introduction 1

Introduction 2

Synaptic Organization of Bullfrog Sympathetic Ganglia 3

Signal Transduction of G-Protein Coupled Receptors 5

Properties of G-Protein Coupled Receptors 5

MAPK Signaling Pathways 7

Signal Transduction of G-Protein Coupled Receptors 10

A) G_s 10

B) $G_{i/o}$ 11

C) $G_{q/11}$ 11

D) Non-Classical Signaling by G-protein Coupled Receptors 13

Signal Transduction of G-Protein Coupled Receptors in Bullfrog Sympathetic Ganglia Neurons 14

a) Luteinizing Hormone Releasing Hormone Receptor 14

b) Muscarinic Receptors 16

c) P2Y Receptors 16

Neurotrophic Regulation of Ion Channel Expression 17

Neurotrophins 17

Acute Actions of Neurotrophins 19

Trophic Actions of GPCR Agonists and Neurotransmitters 20

Trophic Regulation of Na^+ and K^+ Channels 21

Trophic Regulation of Ca^{2+} Channels 22

Ion Channels in Bullfrog Sympathetic Ganglia 25

M-Channels 25

Ca^{2+} Channels 29

Rationale 30

Statement of the Problem 31

References 34

Chapter 2

Examination of the Involvement of Depletion of Phosphatidylinositol 4,5 Bisphosphate in Mediating the Suppression of M-type K^+ Channels by ATP and Muscarine 62

Introduction 63

Methods 64

BFSG Experiments 64

Experiments on tsA-201 and Cos-1 Cells 65

Drugs and Chemicals 66

Results 67

Inhibition of PIP_2 Resynthesis Slows Recovery from g_M Suppression 67

Inhibition of Lipid Cycles Slows Recovery from g_M Suppression 69

PIP_2 Antibodies Attenuate ATP-Induced g_M Suppression 70

Direct Effects of PIP_2 on KCNQ2/3 Channels 71

Effects of PIP_2 on M-Currents in BFSG Cells 72

Investigation of the Limited Effectiveness of PIP_2 on BFSG Neurons 73

Effects of Al^{3+} 74

PIP_2 and Suppression of g_M by Muscarine 75

Discussion 75

Transduction of Agonist-Induced M-channel Suppression 76

Modulation of I_M by PIP_2 79

Possible Role of DAG and Ca^{2+} 81

Conclusion 82

References 115

Chapter 3

Luteinizing Hormone Releasing Hormone-Mediated M-channel Closure Involves Depletion of Phosphatidylinositol 4,5 Bisphosphate 120

Introduction 121

Methods 121

Drugs and Chemicals 121

Results 122
LHRH Suppresses g_M via the Activation of PLC 122
LHRH Suppression of g_M Does Not Involve PKC, IP_3, nor Ca^{2+} 123
Inhibition of PIP_2 Resynthesis Blocks Recovery of g_M 125
Anti-PIP_2 Antibodies Reduce Basal g_M and LHRH-Mediated Suppression 127
Discussion 127
Conclusion 130
References 155

Chapter 4

Neurotrophic Regulation of Ca^{2+} Channels by Luteinizing Hormone Releasing Hormone 159
Introduction 160
Methods 161
Tissue Culture 161
In Vivo Experiments 161
Electrophysiology 162
Chemicals 163
Results 164
Neurotrophic Effect of LHRH on Ca^{2+} Channels 164
LHRH Does Not Alter Voltage-Dependence of Ca^{2+} Current Activation or Inactivation 165
Functional LHRH Receptors Present at Cell Surface After 6d in Culture with LHRH 167
Regulation of N- and L- Type Ca^{2+} Channels by LHRH 168
In Vivo Cut C-fibers 168
Transduction Mechanism for Neurotrophic Regulation of I_{Ca} by LHRH 169
LHRH Induced Changes are Transcription Dependent 169
Inhibition of the Effect of LHRH by Inhibition of Ras 170
Involvement of MEK in the LHRH Response 170

PKC and PLC are Required to Mediate the Effects of LHRH 171
Role of PKA in Mediating the Effects of LHRH.....172
Inhibitors of Src Kinase and PI3 Kinase Do Not
Prevent LHRH-Mediated Changes in I_{Ba}.....172
Direct Activation of PKC, But Not PKA
Potentiates I_{Ba} Density 173
Discussion.....173
Regulation of I_{Ba} by LHRH 174
Signal Transduction Mechanism for Effect of LHRH.....174
Comparison of Action of NGF and LHRH.....176
Physiological Significance.....177
Conclusion 178
References.....209

Chapter 5

General Discussion.....218
Chapter Summaries.....219
M-Type K^+ Channel Regulation by PIP_2.....221
Remaining Questions and Future Directions 224
Regulation of Ca^{2+} Channel Expression by
Luteinizing Hormone Releasing Hormone.....227
Future Directions 229
Conclusions.....230
References.....231

List of Tables

Table	Page
Table 2-1	84
Table 4-1	179

List of Figures

Figure	Page
Figure 1-1	33
Figure 2-1	86
Figure 2-2	88
Figure 2-3	90
Figure 2-4	92
Figure 2-5	94
Figure 2-6	96
Figure 2-7	98
Figure 2-8	100
Figure 2-9	102
Figure 2-10	104
Figure 2-11	106
Figure 2-12	108
Figure 2-13	110
Figure 2-14	112
Figure 2-15	114
Figure 3-1	132
Figure 3-2	134
Figure 3-3	136
Figure 3-4	138
Figure 3-5	140
Figure 3-6	142
Figure 3-7	144
Figure 3-8	146
Figure 3-9	148
Figure 3-10	150
Figure 3-11	152

Figure 3-12 154

Figure 4-1 182
Figure 4-2 184
Figure 4-3 186
Figure 4-4 188
Figure 4-5 190
Figure 4-6 192
Figure 4-7 194
Figure 4-8 196
Figure 4-9 198
Figure 4-10 200
Figure 4-11 202
Figure 4-12 204
Figure 4-13 206
Figure 4-14 208

List of Abbreviations

AP	action potential
α-HFA	α-Hydroxyfarnesylphosphonic acid
4α-phorbol	(4α-phorbol 12-myristate-13-acetate)
ACh	acetylcholine
AKAP150	the multivalent A-kinase-anchoring protein
AMP-PNP	adenosine 5'-(β,γ-imido) triphosphate tetralithium salt hydrate
ATP	adenosine triphosphate
BAPTA	1,2-*bis*(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BFSG	bullfrog paravertebral sympathetic ganglia
cAMP	cyclic adenosine-monophosphate
C_{in}	input capacitance
CRE	cAMP response element-binding protein transcription factor
CREB	cAMP response element-binding protein
CNS	central nervous system
DAG	1,2-diacylglycerol
DAGK	DAG kinase
DMSO	dimethyl sulfoxide
DOG	DAG analogue 1,2-dioctanyl-*sn*-glycerol
DRG	dorsal root ganglia
EGF	epidermal growth factor
EGTA	ethylene glycol-bis-(2-aminoethyl)-N,N', N'-tetraacetic acid
EPSP	excitatory post synaptic potential
ERK	extracellular signal related kinases

FRET	fluorescence resonance energy transfer
FVPP	sodium fluoride, sodium orthovanadate, sodium pyrophosphate
G_α	α-subunit of the G-protein
$G_{\beta\gamma}$	βγ-subunit of the G-protein
GABA	γ-aminobutyric acid
GAP	GTPase-activating protein
g_{Ba}	Ba^{2+} conductance
GDNF	glial-derived neurotrophic factor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factors
GFP	green fluorescent protein
GIRK	G-protein activated inward rectifier K^+ Channels
g_M	M-type K^+ channel conductance
GnRH	gondadotrophin releasing hormone
GnRH-R	gondadotrophin releasing hormone receptor
GPCR	G-protein coupled receptors
G-Protien	guanine nucleotide binding proteins
GRK	G-protein receptor kinase
GTP	guanosine triphosphate
H-89	(N-[2-(p-Bromocinnamylamina)ethyl]-5-isoquinolinesulfonamide-2HCl
I_{Ba}	Ba^{2+} current
I_{Ca}	Ca^{2+} channel current
$I_{Ca, L}$	L-type Ca^{2+} channel current
$I_{Ca, N}$	N-type Ca^{2+} channel current
I_L	leak current
I_M	M-type K^+ channel current
IP_3	inositol trisphosphate
IPSP	inhibitory post-synaptic potential

JNK	c-Jun NH_2-terminal kinase
K_{ATP}	ATP-sensitive K^+ channel
Kir	inwardly rectifying K^+ channel
LHRH	luteinizing hormone releasing hormone
LHRH-R	luteinizing hormone releasing hormone receptor
LTP	long-term potentiation
LY294002	(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MAPK	mitogen-activated protein kinase
M-channel	M-type K^+ channel
ML-7	1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride
MLCK	myosin light chain kinase
NA	Noradrenaline
NGF	nerve growth factor
NMG	N-methyl-D-glucamine
NPY	neuropeptide Y
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
p38	p38 type mitogen activated protein kinase
PA	perillic acid
PACAP	pituitary adenylate cyclase activating peptide
PC	phosphatidylcholine
PC12	cell line derived from rat pheo-chromocytoma cells
PD-98059	2'-Amino-3'-methoxyflavone
PH	pleckstrin homology domain
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3-kinase

PI4K	.....phosphatidylinositol-4-kinase
PIP	.....phosphatidylinositol-4-phosphate
PIP_2	.....phosphatidylinositol 4,5, bisphosphate
PIP_3	.....phosphatidylinositol-3,4,5-trisphosphate
PIPK	.....phosphatidylinositol-4-phosphate-5-kinase
PITP	.....phosphatidylinositol transfer protein
PLC	.....phospholipase C
PKA	.....protein kinase A
PKB	.....protein kinase B
PKC	.....protien kinase C
PMA	.....Phorbol 12-myristate-13-acetate
PTX	.....pertussis toxin
R59022	.....(6-(2-[4-([4-fluorophenyl]phenymethylene)-1-piperidinyl]ethyl)-7-methyl-5H-thiazolo (3,2-a) pyrimidin-5-one)
RGS	.....Regulator of G-protein signaling protein
RL	.....RGS-like domain
RNAi	.....RNA interference
Rp- cAMPS	.....Rp-Adenosine-3',5'-cyclic mononphosphorothioate triethylamine salt
SAPK	.....stress-activated protein kinases
SCG	.....superior cervical ganglia
SH2	.....Src-homology 2 domain
SH3	.....Src-homology 3 domain
SNT	.....suc-associated neurotrophic factor-induced tyrosine-phosphorylated target
Sp-cAMPS	.....Sp-Adenosine-3',5'-cyclic mononphosphorothioate triethylamine salt
SUR	.....sulfonylurea receptor
Trk	.....receptor tyrosine kinase
U-73122	.....1-(6-((17β-3methoxyestra-1,3,5(10)-trien-

		17-yl)amino)hexyl)-1H-pyrrole-2,5-dione
U-73343	.....	1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-pyrrole-2,5-dione
UTP	.....	uridine triphosphate
VGCC	.....	voltage gated Ca^{2+} channel
V_h	.....	holding potential
VIP	.....	vasoactive intestival peptide

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Appropriate functioning of the nervous system requires that each neuron exhibit a specific phenotype. The electrophysiological phenotype of a neuron is primarily determined by the number, properties and location of ion channels in its cell membrane. Electrophysiological properties are important for communication within the neuron (graded local potentials) and for communication between neurons (action potentials and synaptic potentials). Action potentials invading axon terminals induce voltage-gated calcium channels to open, leading to neurotransmitter release from vesicles. Specific neurotransmitter content ensures that each neuron possesses the correct chemical signaling phenotype. Although the genome provides the foundation for the nervous system, it is endogenous and exogenous signals that ensure the correct phenotypic expression of each nerve cell. The roles of these endogenous and exogenous signals have been extensively studied in the developing nervous system. Less is known of their role in the fully mature, developed nervous system. Classically, neurotrophins have been deemed responsible for the trophic regulation of the nervous system, while neurotransmitters were solely responsible for acute communication between neurons. Recent work suggests that this separation of function is not so clearly defined, as neurotrophins and neurotransmitters can act similarly in certain systems. Neurotransmitters which couple to G-protein coupled receptors (GPCR) mediate multiple actions. These effects can be acute, such as the opening or closing of ion channels, or longer lasting, such as initiation of gene transcription and the synthesis of new proteins.

This thesis examines the mechanisms by which neurotransmitters which couple to GPCRs acutely and trophically regulate the electrical properties of fully differentiated, adult neurons of bullfrog sympathetic ganglia (BFSG). It will first examine the mechanisms by which two sympathetic neurotransmitters (ATP and muscarinic acetylcholine) cause acute suppression of M-type K^+ channels. Secondly, it will examine the mechanism of suppression of these channels by the peptide transmitter luteinizing hormone releasing hormone (LHRH) to see if this agonist mediates its effects in a similar manner to other neurotransmitters. Thirdly it will examine the trophic regulation of Ca^{2+} channels by LHRH. These experiments thus provide an opportunity to examine the acute and long term actions of the same neurotransmitter in the same system. Investigation of

the actions of neurotransmitters on the electrical properties of sympathetic neurons is important for furthering the understanding of the autonomic nervous system and may also be of relevance to clinical disorders such as hypertension and congestive heart failure.

Synaptic Organization of Bullfrog Sympathetic Ganglia

The vast majority of experiments described in this thesis have been performed on the paravertebral sympathetic ganglia of the bullfrog (BFSG). The reasons for selecting this experimental system are outlined below.

Bullfrog paravertebral sympathetic ganglia are perhaps the best characterized of all autonomic ganglia. The biophysical properties of the neurons, their peptide and non-peptide transmitter content, and the mechanisms of ganglionic transmission have been extensively studied *in vitro* and *in vivo* (Smith, 1994). There are two principal types of neurons in BFSG: large, exocrine B-cells which project to targets in the skin, and smaller C-cells which project primarily to blood vessels (Horn *et al.*, 1988; Ivanoff & Smith, 1995; Jobling & Horn, 1996; Thorne & Horn, 1997). Pre-ganglionic B- and C- fibers originate from separate groups of neurons in the spinal cord and remain separate as they pass through the paravertebral ganglia (Nishi *et al.*, 1965; Dodd & Horn, 1983a; Horn & Stofer, 1988). B-fibers pass through the IVth, Vth, and VIth roots, while C-fibers emerge in the VIIth, and VIIIth roots (Libet *et al.*, 1968; Horn & Stofer, 1988). In addition to the classical, fast nicotinic excitatory post-synaptic potential (EPSP), three types of slow synaptic potentials can also be evoked by stimulation of pre-ganglionic fibers: the slow EPSP, the late-slow EPSP, and the slow inhibitory post-synaptic potential (IPSP). The slow IPSP and EPSP occur in C-cells and B-cells respectively and are mediated by the muscarinic action of acetylcholine (ACh) (Weight & Padjen, 1973; Dodd & Horn, 1983b; Smith & Weight, 1986). Suppression of a voltage-sensitive M-type K^+ current (I_M) underlies the generation of the slow and late-slow EPSP (Adams *et al.*, 1982a; Adams *et al.*, 1986), which results in increases in excitability of the post-ganglionic neuron. The slow IPSP is generated by activation of an inwardly rectifying K^+ conductance (Dodd & Horn, 1983b; Selyanko *et al.*, 1990b). The late-slow EPSP which occurs in both B- and C-cells (Jan *et al.*, 1979) is mediated by the peptide transmitter luteinizing hormone-releasing hormone (LHRH; probably chicken II gondadotrophin

releasing hormone (GnRH)) (Jan *et al.*, 1979; Jan *et al.*, 1980b; Jones, 1987b), which is released from pre-ganglionic C-fibers, (Jan *et al.*, 1980b). As LHRH can diffuse out of the C-fiber/C-cell synaptic cleft to activate receptors on B-cells, it allows for an interaction of the C-fiber and B-fiber systems within the paravertebral sympathetic ganglia (Ivanoff & Smith, 1995; Ivanoff & Smith, 1997; Ford *et al.*, 2000). In addition to ACh and LHRH, a variety of peptide (e.g. neuropeptide Y, Substance P, somatostatin) and non-peptide (e.g. ATP, adrenaline) neurotransmitters and neuromodulators also alter a variety of ionic conductances within BFSG (Smith, 1994).

In addition to their well characterized anatomical and biophysical properties, BFSG possess several unique characteristics which make them an excellent model in which to investigate both short- and long-term regulation of ion channels by neurotransmitters. (1) BFSG contain only two neuron types: B- and C- cells (Horn *et al.*, 1988; Ivanoff & Smith, 1995), each of which are easily identifiable *in vitro* and in culture on the basis of size and the consistency of their electrophysiological properties (Adams *et al.*, 1986; Jassar *et al.*, 1993; Smith, 1994). This is unlike mammalian ganglia. For instance, dorsal root ganglia (DRG) possess as many as 43 different neuronal subtypes (Lewin, 1996). This complicates the use of cultures of DRG neurons when the same neuronal subtype is to be studied under different experimental conditions. (2) To study the trophic actions of a neurotransmitter, an appropriate culture system is required, which is free from the influence of other trophic factors. Adult BFSG neurons can be maintained in a serum-free low-density, defined-medium culture system which is free from the complicating effects of exogenously-applied or endogenous neurotrophins for $\geq$14 days (Lei *et al.*, 1997). While in culture, BFSG neurons remain identifiable on the basis of size (Lei *et al.*, 1997) allowing for consistent examination of the same cell type. (3) While studying the possible trophic actions of LHRH *in vitro* is important, it is also nessesary to test the effects *in vivo*. LHRH is solely released from pre-ganglionic C-fibers in BFSG (Jan *et al.*, 1979; Jan *et al.*, 1980b; Jobling & Horn, 1996; Thorne & Horn, 1997). By cutting these fibers, it is possible to remove the tonic influence of LHRH, and thus examine the role that LHRH plays *in vivo* in the regulation of ion channels. (4) As mentioned above, muscarinic agonists produce different effects on different cell types in BFSG. Because cell types are easily identifiable after dissociation,

it is possible to consistently and separately examine the two different effects of muscarine, namely the suppression of M-type K^+ channels in B-cells and the activation of the inwardly rectifying K^+ conductance in C-cells. In addition, several other neuromodulators are present within BFSG which also modulate M-channels. This allows for comparisons to be made between the effects of different neurotransmitters on these channels.

Signal Transduction of G-Protein Coupled Receptors

Since this thesis deals with the actions mediated by GPCRs, their basic properties and mechanisms of action will be described.

Properties of G-Protein Coupled Receptors

GPCRs are a superfamily of 7-transmembrane spanning receptors which couple to heterotrimeric guanine nucleotide-binding proteins (G-protein) and transduce the signal of a wide variety of neurotransmitters and hormones. Activation of a GPCR by an agonist induces a conformational change in the receptor, promoting the interaction of the GPCR and G-protein (Gether & Kobilka, 1998; Gether, 2000; Yeagle & Albert, 2003). This leads to the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α-subunit of the G-protein (G_α) and dissociation of the G-protein βγ heterodimer subunit ($G_{\beta\gamma}$) (Neer, 1995; Surya *et al.*, 1998; Surya *et al.*, 1998). Active GTP-bound G_α or dimers of $G_{\beta\gamma}$ subunits are then free to interact with, and activate or inactivate various downstream intracellular effectors. Inactivation is determined by the intrinsic GTPase activity of G_α which hydrolyzes GTP to GDP, promoting the re-association of G_α and $G_{\beta\gamma}$. G_α subunits are inefficient GTPases, with the intrinsic GTPase activity of purified G_α subunits being much less than the observed rate of G-protein deactivation in cellular contexts (Hollinger & Hepler, 2002). Regulators of G-protein signaling (RGS) are proteins which were first identified as negative regulators of G-protein signaling (Hollinger & Hepler, 2002). RGS proteins are a family of over thirty distinct proteins all containing the signature RGS or RGS-like (RL) domain (Hollinger & Hepler, 2002). These proteins act as GTPase-activating proteins (GAPs; discussed

below) which greatly accelerate the rate of G_α induced GTP hydrolysis (up to 1000 fold), by binding directly to the active GTP bound forms of $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\alpha_{i/o}$ (Dohlman & Thorner, 1997; Hepler, 1999; Hollinger & Hepler, 2002). Alternatively, RGS proteins may also act as competitive antagonists by preventing GTP bound G_α from interacting with effector molecules (Berman & Gilman, 1998).

Prolonged agonist application leads to receptor desensitization. There are two forms of desensitization. Heterologous desensitization occurs by phosphorylation of GPCRs by protein kinase A (PKA) and protein kinase C (PKC) (Benovic *et al.*, 1985; Pitcher *et al.*, 1992). This type of desensitization involves a feedback from the second messenger activated kinases reducing the activity of the receptor and inhibiting production of the second messenger. Heterologous desensitization is non-specific, occurring when any signal activates intracellular kinases. These kinases phosphorylate serine residues on the third intracellular loop or the C-terminus tail of the GPCR leading to receptor uncoupling from G-proteins. Homologous desensitization, which is the more common mechanism for agonist-induced GPCR desensitization, is specific for the activated receptor and involves uncoupling of the receptor from the G-protein, sequestration of the receptor from the membrane and internalization (Freedman & Lefkowitz, 1996). This process involves phosphorylation of the agonist-occupied receptor by G-protein receptor kinases (GRKs). GRKs are a family of seven serine/threonine kinases (Kohout & Lefkowitz, 2003) which are constitutively associated with the plasma membrane (GRK1, GRK4, GRK5, GRK6, and GRK7) or are recruited to the membrane after GPCR activation (GRK2, and GRK3) (Kohout & Lefkowitz, 2003; and references within). Agonist bound GPCR assume a configuration which promotes one or more GRKs to bind. Bound GRKs phosphorylate GPCRs at both serine and threonine residues localized within either the third intracellular loop or carboxyl-terminal tail domains (Ferguson, 2001). The phosphorylation of these specific residues on the receptors promotes the binding of the protein arrestin which inhibits G-protein coupling (Lohse *et al.*, 1990; Lohse *et al.*, 1992). GPCR-bound GRKs can be positively or negatively regulated by phosphorylation by second messenger kinases (Kohout & Lefkowitz, 2003).

Arrestins constitute a family of four proteins which are classified into two categories, depending on their associated receptors and anatomical distribution as visual arrestins and β-arrestins (Ferguson, 2001; and the references contained within). Visual arrestin possesses a specific pattern of distribution which is unlike β-arrestin 1 and 2, being ubiquitous and coupling to multiple different GPCRs (Attramadal *et al.*, 1992; Bohn *et al.*, 2000). Arrestins bind preferentially to GRK-phosphorylated GPCR, as opposed to second messenger-phosphorylated receptors, or non-phosphorylated receptors (Lohse *et al.*, 1990; Lohse *et al.*, 1992). Arrestins also bind clathrin (Goodman, Jr. *et al.*, 1996), which via the AP-2 heterotetrameric adaptor complex, targets GPCR to clathrin-coated pits (Zhang *et al.*, 1996; Laporte *et al.*, 1999). This leads to internalization by clathrin-coated vesicles (Ferguson, 2001), and a reduction in activated receptors from the membrane surface. The stoichiometric differences in GRK-mediated phosphorylation of GPCR subtypes may underlie the affinity of receptor/arrestin complexes (Oakley *et al.*, 1999). This may contribute to differences in intracellular trafficking and signaling of desensitized receptors (Ferguson, 2001).

Receptor binding to specific G-proteins determines the signaling pathways activated. There are currently 20 known G_{α}, 6 G_{β}, and 11 G_{γ} subunits (Neves *et al.*, 2002). The G_{α} subunits have been divided into four families: $G\alpha_s$ $G\alpha_{i/o}$, $G\alpha_{q/11}$ $G\alpha_{12/13}$. Substantial work has been devoted to determining the signal transduction pathways of GPCR, and is too vast a topic to be discussed here. This section will briefly review those transduction pathways relating to this thesis. Emphasis will be placed upon activation of the Ras/mitogen-activated protein kinase (MAPK) pathways by GPCRs, and the signaling pathways used by specific neurotransmitters that couple to GPCR in BFSG.

MAPK Signaling Pathway

There exist three families of mitogen-activated protein kinases: (1) extracellular signal related kinases (ERK1 and ERK2), (2) p38 MAPKs (p38α, p38β, p38γ, and p38δ); and (3) c-Jun-NH_2-terminal kinases (JNK1, JNK2, and JNK) (Johnson & Lapadat, 2002). JNK and p38 kinases are members of the stress-activated protein kinases (SAPKs) and are primarily involved in the arresting of cell growth, apoptosis, and responses to inflammation and environmental and hormonal stresses (Kyriakis & Avruch, 1996). The

ERK pathway is primarily involved in the regulation of mitosis/meiosis and postmitotic functions of differentiated cells (Pearson *et al.*, 2001; Johnson & Lapadat, 2002). In BFSG, the trophic regulation of Ca^{2+} channels by nerve growth factor involves the Ras/ERK pathway (Lei *et al.*, 1998). As this thesis examines the trophic regulation of Ca^{2+} channels by LHRH, it is the ERK pathway which is hypothesized to be similarly involved. Thus it is this pathway which will be primarily discussed here.

MAPKs are activated by a series of sequentially activated kinases which successively phosphorylate and activate downstream components. Distinct activation of certain pathways depends upon the nature of the stimulus and the cell type. The idea of 'cellular context' is important as similar signals can activate different pathways in different cell types depending upon the metabolic and developmental status of the cell. This complicates the elucidation of signal transduction pathways as not all proteins/pathways behave in an identical manner in all cells. A high degree of similarity exists between the different members of the MAPKs in terms of their activation, organization and sequence homology of proteins within their pathways (~60% sequence homology exists between ERKs, JNKs, and p38 kinases) (Pouyssegur *et al.*, 2002). Fidelity of signaling to prevent inappropriate cross talk between different MAPKs is maintained by two general mechanisms (Pouyssegur *et al.*, 2002): (1) scaffolding proteins which assemble specific kinases into a complex (Whitmarsh & Davis, 1998), and (2) specific docking sites on each of the MAPK members which ensures the correct binding and activation of certain kinases (Tanoue *et al.*, 2000).

The basic signaling pathway leading to the stimulation of ERK is activation of the small monomeric GTP binding protein Ras ($p21^{ras}$/p21Ras). Ras recruits the serine/threonine MAPK kinase kinase (Raf) to the plasma membrane where it is activated, and in turn activates a threonine/tyrosine MAPK kinase (MEK), subsequently activating MAPK (ERK) (Seger & Krebs, 1995; Lewis *et al.*, 1998; Kolch, 2000). Specificity is maintained as MEK is dually phosphorylated on two serine residues by Raf and ERK is dually phosphorylated on a tyrosine and threonine residue (TEY sequence) by MEK (Pouyssegur *et al.*, 2002).

Once activated, ERK translocates to the nucleus to phosphorylate and activate nuclear transcriptions factors such as the cAMP response element-binding protein

(CREB) transcription factor (CRE), Ets, Elk, Myc as well as other substrates such as p90-RSK (ribosomal S6 kinase) which are involved in DNA transcription and cell growth and division (Impey *et al.*, 1998; Lewis *et al.*, 1998; Pearson *et al.*, 2001). Different signals cause ERK to be redistributed within the cell in different phases. This leads to differing 'strengths' of ERK signaling (Pouyssegur *et al.*, 2002). Mitogenic stimulation induces a rapid entry of ERK into the nucleus, without depleting the cytoplasmic pool, which is followed after several hours of stimulation by an emptying of the cytoplasmic pool and an accumulation in the nucleus (Volmat *et al.*, 2001; Pouyssegur *et al.*, 2002). Non-mitogenic signaling induces only the initial ERK entry into the nucleus, without the secondary accumulation (Volmat *et al.*, 2001; Pouyssegur *et al.*, 2002). These differences in ERK nuclear accumulation may partially account for differences in effects caused by different stimuli, all of which commonly activate ERK.

Signal transduction within the ERK pathway is complex, and is regulated by multiple pathways and proteins. Such redundancy within the system may allow for subtle differences in signaling, depending the signal and cell type. The Ras superfamily of small GTPases includes over 150 members and contains six subfamilies: Ras, Rho, Ran, Rab, Arf and Kir/Rem/Rad (Ehrhardt *et al.*, 2002). The Ras subfamily is comprised of 13 members, classified into 5 groups: p21Ras (H-Ras, K-Ras, and N-Ras), M-Ras, R-Ras, Rap and Ral (Ehrhardt *et al.*, 2002). Activation of Ras requires translocation from the cytoplasm to the cell membrane. It is at this point that GTP/GDP exchange occurs which activates Ras. Members of this subfamily contain a CaaX motif. This is the target of enzyme-induced post-translational isoprenylation (either farnesylation or geranylgeranylation) which functions to anchor Ras to the plasma membrane (Casey, 1995). Ras subfamily members, like other G-proteins are positively regulated by other signaling molecules. Inactive GDP-bound Ras proteins are activated by guanine-nucleotide exchange factors (GEFs), which promote the exchange of GTP for GDP. GEFs have the abiltity to activate either individual, or multiple Ras family/superfamily members, depending on the specific GEF examined (Ehrhardt *et al.*, 2002). Family members of GEFs which activate Ras include proteins such as SOS1/2, RasGRP, RasGRF, and Epac1/2 (Ehrhardt *et al.*, 2002). Additionally, GEFs are activated by a variety of additional signals, such as changes in $[Ca^{2+}]_i$, phosphorylation, cyclic

nucleotides and diacylglycerol (Ehrhardt *et al.*, 2002). Inactivation, or negative regulation, of G-proteins is promoted by GTPase activating proteins (GAPs), which enhance the GTPase activity of G-proteins (Donovan *et al.*, 2002). Multiple members of GAP family exist, each with the ability to regulate various G-proteins, such as heterotrimeric G-proteins (RGS) or members of the Ras family (the RAS GAP, p120GAP) (Donovan *et al.*, 2002).

Ras family members have a wide variety of actions on effector molecules, which include such proteins as GEFs or GAPs for other Ras superfamily members, as well as protein and lipid kinases such as phosphatidylinositol-3-kinase (PI3K) (Rodriguez-Viciana *et al.*, 1994) and phospholipase Cε (Song *et al.*, 2001; Kelley *et al.*, 2001). However, in regards to this thesis, it is important to note that p21Ras, is the primary effector for Raf. Three Raf family members are currently known: A-Raf, B-Raf, and Raf-1. Differences in signaling also occur at this level, as Ras binding alone is sufficient to activate B-Raf (the major Raf isoform in neurons), but not Raf-1 or A-Raf (Marais *et al.*, 1997).

There are three 'general' mechanisms by which GPCRs can activate ERK: (1) via second messenger or classical transduction mechanisms (i.e. Ca^{2+}, PKA, PKC), (2) via the transactivation of growth factor, tyrosine kinase receptors, and (3) via signaling by β-arrestin-induced scaffolding of components of the ERK kinase cascade. These different mechanisms will be discussed below in the context of GPCR signaling.

Signal Transduction of G-Protein Coupled Receptors

a) G_s

Activation of $G_\alpha s$ leads to the stimulation of adenylyl cyclase which produces cAMP (Birnbaumer, 1992). In its classical role, cAMP can then act as a second messenger to activate PKA. Additionally, cAMP can also activate the cAMP-responsive RAS-GEF, Epac, which leads to the activation of Rap-1 (a member of the Ras subfamily, see above) (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Rap-1 may also be directly activated by PKA (Vossler *et al.*, 1997; Grewal *et al.*, 2000). Rap-1 proceeds to activate the downstream molecule Raf. Different cell types possess different isoforms of Raf. In neurons and PC12 cells (a cell line derived from rat pheochromocytoma cells), B-Raf is

present, and its activation leads to the activation of ERK (Erhardt *et al.*, 1995; Vossler *et al.*, 1997; Dugan *et al.*, 1999; Grewal *et al.*, 1999; Grewal *et al.*, 2000) . Non-neuronal cells possess Raf-1 which is inhibited by the cAMP-pathway via Rap-1; this activation leads to the inhibition of ERK (Wu *et al.*, 1993; Cook & McCormick, 1993; Hafner *et al.*, 1994; D'Angelo *et al.*, 1997; Dugan *et al.*, 1999; Impey *et al.*, 1999). The effect of cAMP on ERK is thus cell type dependent. Additionally, cAMP has been reported to directly activate Ras (Ambrosini *et al.*, 2000; Tsygankova *et al.*, 2000), further implicating this pathway in the activation of ERK.

b) $G_{i/o}$

$G_{\alpha}i$ has an inhibitory effect on adenylyl cyclase activity (Birnbaumer, 1992) and can inhibit the activation of ERK by a mechanism opposite to that of $G_{\alpha}s$. $G_{\alpha}i$ and $G_{\alpha}o$ can directly interact with Rap-1-GAP causing either activation or inhibition of ERK, depending on the cell type studied (Jordan *et al.*, 1999; Mochizuki *et al.*, 1999). $G_{\alpha o}$ has also been reported to activate B-Raf, the major isoform of Raf in neurons, which leads to the activation of ERK (Antonelli *et al.*, 2000).

$G_{\alpha}i/o$ coupled receptors are well known to cause activation of ERK via processes mediated by the $G_{\beta\gamma}$ subunits (Pierce *et al.*, 2001; Luttrell, 2002). There exist several pathways by which $\beta\gamma$ dimmers can activate ERK: transactivation of growth factor receptors, which further recruit intracellular scaffolding proteins such as Grb and SOS to activate ERK (Daub *et al.*, 1996; Ferguson, 2003); activation of Ras-GRF, a GEF for Ras which leads to ERK activation (Crespo *et al.*, 1994; Koch *et al.*, 1994; Mattingly & Macara, 1996), and activation of phosphatidylinositol 3 kinase γ (PI3Kγ), (Hawes *et al.*, 1996; Stephens *et al.*, 1997). PI3Kγ activation can stimulate ERK through multiple pathways, including activation of PKC (Takeda *et al.*, 1999) or phosphorylation of upstream pathways known to activate the Ras/ERK cascade (Bondeva *et al.*, 1998).

c) $G_{q/11}$

$G\alpha q/11$ activation stimulates the ERK cascade primarily by activating the membrane associated phospholipase C (PLC). The PLC family enzymes are comprised of 11

isozymes which can be divided into four classes: PLCβ(1-4), PLCγ(1-2), PLCδ, and PLCε (Fukami, 2002). PLCβ proteins can be activated by both the α-subunits of $G_{q/11}$ proteins or the βγ-subunits of either $G_{q/11}$ or $G_{i/o}$ proteins, depending on the subfamily member (Fukami, 2002), and the references listed within). PLCγ are characterized by two SH2 and one SH3 domain which allows for their activation by receptor and non-receptor tyrosine kinases (Fukami, 2002) (see below), while PLCδ and PLCε are regulated by Ca^{2+} and Ras respectively (Fukami, 2002).

PLC mediates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to the cytosolic product inositol 1,4,5 triphosphate (IP_3) and the membrane bound product 1,2-diacylglycerol (DAG) (Voet & Voet, 1995). The resynthesis of PIP_2 following PLC-mediated hydrolysis follows a lengthy biochemical pathway (Fig 1-1). DAG kinase (DAGK) converts DAG to phosphatidic acid which is converted to CDP-DAG by CTP-phosphatidate cytidyl transferase (Voet & Voet, 1995). IP_3 is dephosphorylated sequentially to IP_2, IP, and inositol by IP_3-, IP_2-, and IP_1-phosphatases (Voet & Voet, 1995). PI synthase combines CDP-DAG and inositol to phosphatidylinositol (PI), which is phosphorylated sequentially to PIP and PIP_2 by PI4-kinase (PI4K) and PI4P5-kinase (PIPK) (Voet & Voet, 1995), to eventually complete the cycle back to PIP_2. The synthesis of PIP_2 is also dependent upon the phosphatidylinositol transfer proteins (PITP) family of proteins. These proteins transport phosphatidylinositol (PI) and phosphatidylcholine (PC) (Allen-Baume *et al.*, 2002) to cellular compartments where they can be further phosphorylated to PIP_2 by PI4K and PIPK (Aikawa *et al.*, 1999). Durring PLC activation, PITPs providing a continued source of plasma membrane PIP_2 by the transfer of PI from intracellular pools (Thomas *et al.*, 1993; Kauffmann-Zeh *et al.*, 1995; Whatmore *et al.*, 1999). PIP_2 can be further phosphorylated to PIP_3 by PI3K, leading to the activation of the protein kinase B (PKB)/Akt pathway (Czech, 2000; Cantrell, 2001)

The production of IP_3 can lead to the mobilization of free intracellular Ca^{2+} from IP_3-sensitive stores. Increases in $[Ca^{2+}]_i$ leads to ERK activation through various pathways. Free Ca^{2+} can activate Ras and ERK via CaM Kinase II-induced inhibition of Ras-GTPase-activating protein (Ras-GAP) (Chen *et al.*, 1998). Ca^{2+} also leads to the activation of the protein tyrosine kinase (Pyk2) which leads to Ras-dependent ERK

activation by the recruitment of the nonreceptor kinase Src (Lev *et al.*, 1995; Dikic *et al.*, 1996; Della Rocca *et al.*, 1999). Additionally, Ca^{2+} can lead to the activation of Ras by activating several Ras-GEFs, such as the exchange factor Ras-GRF (Farnsworth *et al.*, 1995) or Ras-GRP, a Ras nucleotide releasing protein, which is activated by Ca^{2+} and DAG (Ebinu *et al.*, 1998).

Ca^{2+} and/or DAG can activate protein kinase C (PKC) (Nishizuka, 1992). This can lead to the activation of ERK in a Ras-independent manner, as PKCα can directly stimulate the activation of Raf by direct phosphorylation which in turn initiates cascades to activate ERK (Kolch *et al.*, 1993).

d) *Non-Classical Signaling by G-Protein Coupled Receptors*

GPCR may also signal in a non-classical manner by the binding of β-Arrestins (Pierce *et al.*, 2001; Luttrell, 2002; Grimes & Miettinen, 2003). β-Arrestins binding to GPCRs block receptor/G-protein interactions (see above). This can promote the binding of Src which confers tyrosine kinase activity upon the receptor-arrestin complex (Luttrell *et al.*, 1999). This leads to recruitment of the components of the ERK cascade (Raf, Mek, ERK) (Luttrell, 2002). Arrestin binding also leads to internalization of the GPCR (see above). This process can also activate ERK (McDonald & Lefkowitz, 2001). β-Arrestins also activate other members of the MAPK family such as members of the JNK familiy, in a manner similar to the activation of ERK (McDonald *et al.*, 2000).

GPCRs have recently been discovered on nuclear membranes where they can functionally bind agonists and couple to signaling mechanisms (Bhattacharya *et al.*, 1999; Boivin *et al.*, 2003). This suggests that GPCR signaling may not be limited to the plasma membrane. The activated receptors may be derived from internalized, agonist bound, plasma-membrane receptors which have translocated from the nuclear membrane (Lu *et al.*, 1998). Additionally, they may be natively expressed on nuclear membranes where they are activated by intracellularly produced agonists acting in a 'intracrine' manner (Boivin *et al.*, 2003).

Signal Transduction of G-Protein Coupled Receptors in Bullfrog Sympathetic Ganglia Neurons

a) Luteinizing Hormone Releasing Hormone Receptor

Gonadotropin hormone-releasing hormone (GnRH), also refered to as luteinizing hormone releasing hormone (LHRH), belongs to a family of decapeptides conserved throughout vertebrate evolution (Montaner *et al.*, 2001). To date 14 forms have been characterized (Montaner *et al.*, 2001). Forms of GnRH are differentially distributed in pituitary and other neural and peripheral tissues with chicken II-GnRH being ubiquitously distributed across vertebrate classes (Klausen *et al.*, 2002). As already mentioned a peptide resembling LHRH mediates the late slow excitatory post-synaptic potential (late slow e.p.s.p.) in BFSG (Jan *et al.*, 1979; Jan *et al.*, 1980a; Jan *et al.*, 1980b; Jan & Jan, 1982). Chicken II-GnRH is ~100 times more potent than salmon GnRH and >1000 times more potent than other naturally occurring GnRH analogs in BFSG suggesting that chicken II-GnRH (chicken II-LHRH) may be the endogenous transmitter mediating this effect (Jones, 1987b). The GnRH-receptor (LHRH-R) has been identified from both mammalian and non-mammalian sources as having the typical 7-transmembrane spanning segments of the G-protein coupled receptor (GPCR) family (Eidne et al 1992; Tsutsumi et al 1992). Three types of GnRH receptors have been discovered to date. The type II GnRH-receptor is selective for chicken II-GnRH (Troskie *et al.*, 1997; Millar *et al.*, 2001), also see (Klausen *et al.*, 2002) suggesting that it is this receptor which mediates the effects of LHRH in BFSG.

Cloning of non-mammalian GnRH receptors from goldfish, chicken and the frog (Troskie *et al.*, 1998; Illing *et al.*, 1999) have shown the presence of a C-terminal tail, which is not present in mammalian forms of the receptor. The mammalian GnRH-R, which lacks the C-terminal tail, does not rapidly internalize upon continued agonist exposure (Willars *et al.*, 1998a). Furthermore, non-mammalian GnRH-Rs (including the type II-GnRH-R) which do possess a C-terminal tail and mammalian GnRH-Rs chimerized with a C-terminal tail from catfish, rapidly desensitize/internalize upon agonist exposure (Heding *et al.*, 1998; Lin *et al.*, 1998).

A single GPCR type can activate multiple second messenger pathways (i.e. dual signaling). This signaling is dependent on both receptor/G-protein affinity (Ashkenazi *et*

al., 1987), and receptor density (Zhu *et al.*, 1994). The LHRH-R, depending on the cell type, can also activate different G proteins/signal transduction pathways (Conn *et al.*, 1979; Kuphal *et al.*, 1994). $G_{q/11}$, G_s and G_i proteins have all been reported to couple to, and mediate the actions of, the various isoforms of the GnRH receptor in a variety of cells and cell lines (Hsieh & Martin, 1992; Hawes *et al.*, 1993; Janovick & Conn, 1994; Conn & Crowley, Jr., 1994; Stanislaus *et al.*, 1997; Stanislaus *et al.*, 1998a; Stanislaus *et al.*, 1998b; Ulloa-Aguirre *et al.*, 1998; Naor *et al.*, 2000; Klausen *et al.*, 2002). While at the present time there have been no studies in BFSG which have identified the G-proteins coupling to the LHRH-R, a variety of signaling pathways may be accessed by LHRH in BFSG. It is known that in BFSG LHRH increases IP_3 turnover and intracellular Ca^{2+} concentration (Pfaffinger *et al.*, 1988), furthering the idea that the LHRH-R couples to $G\alpha_{q/11}$ proteins leading to the activation of PLC. $G\alpha_{q/11}$ has been accepted as the primary G-protein which couples to the GnRH-R in most systems (Hsieh & Martin, 1992; Stojilkovic & Catt, 1995; Naor *et al.*, 1998). However multiple studies have identified other G-proteins as mediating the actions of the GnRH, including $G\alpha_{i/o}$ (Hawes *et al.*, 1993; Imai *et al.*, 1996) and $G\alpha_s$ (Hawes *et al.*, 1993; Janovick & Conn, 1993; Kuphal *et al.*, 1994). However, in catfish and goldfish pituitary cells, chicken II-GnRH did not alter cAMP levels (Chang *et al.*, 1992; Jobin *et al.*, 1996; Rebers *et al.*, 2000), leading to the suggestion that the cAMP system may be important as a modulator, rather than a direct effector of GnRH signaling pathways (Klausen *et al.*, 2002).

GnRH activates ERK in a variety of cells and cell lines (Mitchell *et al.*, 1994; Sim *et al.*, 1995; Roberson *et al.*, 1995; Sundaresan *et al.*, 1996; Reiss *et al.*, 1997; Mulvaney *et al.*, 1999; Han & Conn, 1999; Naor *et al.*, 2000; Benard *et al.*, 2001; Klausen *et al.*, 2002). The activation of ERK by GnRH is both PKC and MEK dependent, with tyrosine phosphorylation and Ca^{2+} dependent components also possibly participating (Sundaresan *et al.*, 1996; Reiss *et al.*, 1997; Call & Wolfe, 1999; Mulvaney *et al.*, 1999; Benard *et al.*, 2001). Two pathways are believed to exist for the activation of ERK by GnRH: (1) via the activation of Raf-1 by PKC, and (2) via Src and Ras (i.e. the transactivation of growth factor receptors) (Benard *et al.*, 2001; Klausen *et al.*, 2002). The presence of the first pathway in BFSG is questionable, as B-Raf, not Raf-1, is the primary Raf isoform present in neurons (see above).

The possible multiple signaling pathways available for the LHRH-R to signal in BFSG suggest that there may be alternate pathways leading to the activation of ERK. At this time the specific pathways by which LHRH activates ERK in BFSG have not been examined.

b) Muscarinic Receptors

Acetylcholine (ACh) acts on two types of receptors: nicotinic and muscarinic. In BFSG, stimulation of nicotinic receptors generates a fast e.p.s.p. which lasts from 20-200msec. Fast e.p.s.p.s are often large enough to generate an action potential in post-synaptic neurons. Stimulation of muscarinic receptors in sympathetic ganglia and elsewhere in the nervous system is associated with slower neuromodulatory effects. This can include modulation of Ca^{2+} or K^+ channels and/or the generation of slow excitatory or inhibitiory post-synaptic potentials, depending on the cell type.

Gene cloning has identified five types of muscarinic receptors, with four of these being distinguishable functionally/pharmacologically (Rang *et al.*, 1999). The M_1-type muscarinic receptor is found primarily on central and peripheral neurons and is coupled to $G_\alpha q/11$ proteins (Rang *et al.*, 1999). M_1 receptors mediate their effects through the inositol phosphate pathway by activating PLCβ to hydrolyze PIP_2 into IP_3 and DAG (see above). Muscarinic depolarization in mammalian autonomic ganglia is mediated by M_1-receptors (Brown *et al.*, 1980; Ashe & Yarosh, 1984; Newberry *et al.*, 1985; North *et al.*, 1985). However, pharmacological examination of this depolarization in the frog suggests that a different muscarinic receptor mediates this response (Yavari & Weight, 1987).

c) P_2Y Receptors

Receptors for extracellular nucleotides are classified into two categories: P2X, which are ATP-gated cation channels and P2Y, which are GPCR activated by purine or pyrimidine nucleotides (Abbracchio & Burnstock, 1994). P2Y receptors are known to be coupled to phospholipid turnover and intracellular Ca^{2+} mobilization indicating the coupling of P2Y receptors to $G\alpha_{q/11}$ (Harden *et al.*, 1995; von Kugelgen & Wetter, 2000; Sak & Webb, 2002). Additionally, other subtypes of P2Y receptors are also known to

couple to pathways activated by $G\alpha_{i/o}$ and $G\alpha_s$ (Communi *et al.*, 1996; Communi *et al.*, 1997; Mosbacher *et al.*, 1998). In superior cervical ganglia (SCG) neurons, $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors have been reported to couple to both pertussis toxin (PTX)-sensitive (presumably $G\alpha_{i/o}$) and -insensitive (presumably $G\alpha_{q/11}$) G-proteins, indicating 'promiscuous' coupling of P2Y receptors to multiple classes of G-protein (Brown *et al.*, 2000). Activation of P2Y receptors in BFSG mediates a slow depolarization that is sensitive to PLC inhibitors and also results in the mobilization of intracellular Ca^{2+} (Stemkowski *et al.*, 2002), suggesting that the P2Y receptors in BFSG also couple to $G\alpha_{q/11}$ proteins.

Neurotrophic Regulation of Ion Channel Expression

One aim of this thesis is to determine whether a GPCR neurotransmitter can act in a tropic manner to regulate the expression of Ca^{2+} channels within BFSG. In other words, it examines if a neurotransmitter may function as a neurotropic factor (neurotrophin). This section will provide an overview of neurotrophins and their actions. It will also discuss what is known of the trophic actions of GPCR agonists and neurotransmitters, molecules not classically known for trophic actions. Finally this section will outline the current state of knowledge regarding trophic regulation of ion channels.

Neurotrophins

Neurotrophins are secretory proteins which regulate neurite outgrowth, neuronal survival and differentiation, and phenotypic maintenance of mature neurons (Barde, 1989). Four types of neurotrophins have been identified, all of which are widely expressed throughout the central nervous system (CNS): brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and nerve growth factor (NGF) (Lewin & Barde, 1996). Neurotrophins mediate their effects by the binding to the family of high affinity, growth factor, receptor tyrosine kinases (TrkA, TrkB, TrkC), with NGF binding to TrkA, BDNF and NT-4/5 binding to TrkB, and NT-3 binding to TrkC (Chao, 1992). Additionally, the non-selective, low affinity p75 receptor

can also mediate signaling or modulate the signaling of the other Trk receptors (Segal & Greenberg, 1996; Friedman & Greene, 1999). As opposed to most neurons which depend on BDNF, NT-3 or NT-4/5, sympathetic neurons, which are a subpopulation of neural crest-derived neurons, and cholinergic neurons in the basal forebrain and striatum are dependent upon NGF (Lindsay, 1996). NGF is necessary for the survival of sympathetic and nociceptive, sensory neurons during their development (Levi-Montalcini & Angeletti, 1968; Lewin, 1996). However, during maturation, these neurons lose their dependence upon NGF for survival. At this point, NGF functions to specify and maintain neuronal phenotypes (Lewin *et al.*, 1992; Lewin, 1996; Lindsay, 1996).

NGF binding induces dimerization of the TrkA receptor (Jing *et al.*, 1992). This leads to autophosphorylation of tyrosine residues on the receptor (Schlessinger & Ullrich, 1992). These phosphorylated regions serve as binding areas for Src-homology 2 and 3 (SH2 and SH3) proteins which act as scaffolding proteins to recruit cytoplasmic proteins involved in transduction of the signal. At least four separate signal transduction pathways are known for NGF: phosphatidylinositol-3-kinase (PI3K) (Carter & Downes, 1992), PLC-γ (Traynor *et al.*, 1982), Ras (Ohmichi *et al.*, 1994; McCormick, 1994), and suc-associated neurotrophic factor-induced tyrosine-phosphorylated target (SNT) (Rabin *et al.*, 1993). Tyrosine residues 490 in the juxtamembrane region and 790 in the cytoplasmic domain of Trk receptors are key for the binding of scaffolding proteins (Kaplan & Miller, 2000; Lee *et al.*, 2002). Shc binding at Tyr-490 acts as an adaptor for the recruitment of other proteins which lead to activation of the Ras/ERK or PI3K pathways. PLCγ binds at Tyr-790 where it is activated and mediates its effects. Each of these processes can activate a variety of downstream pathways affecting neuronal regulation, differentiation, and survival.

NGF promotes the activation of ERK in PC12 cells and sympathetic neurons. This occurs in two phases: the initial rapid activation of ERK, which is Ras dependent, and the sustained activation which is dependent upon Rap-1 (York *et al.*, 1998). These processes are activated by classical downstream signaling from the Trk receptor. As described above, Trk receptors can also be transactivated by GPCRs (Lee & Chao, 2001; Lee *et al.*, 2002). Most of these effects have been examined in regards to the transactivation of the epidermal growth factor (EGF) receptor. Notable differences occur

between the transactivation of Trk and EGF receptors (Lee & Chao, 2001; Lee *et al.*, 2002). Firstly, transactivation of Trk receptors occurs with a slower time course (60-90min) compared to the EGF receptor (within minutes). Secondly, many GPCR agonists which transactivate the EGF receptor do not transactivate Trk receptors in PC12 cells, even though their functional receptors are present. This includes agonists such as bradykinin, ATP and carbachol (Lee & Chao, 2001). Thirdly, transactivation of Trk receptors, at least by adenosine, results in the long-lasting activation of the PI3K, but not the ERK pathway (Lee *et al.*, 2002).

Acute Action of Neurotrophins

The distinction between what is a 'neurotransmitter' and what is a 'neurotrophic factor' has become less defined in recent years because of the findings that neurotrophins have fast actions with time courses similar to neurotransmitters. Neurotrophins can be released in an activity-dependent manner (Balkowiec & Katz, 2002) and have effects which include modulation of synaptic transmission and acute non-trophic regulation of ion channels, both of which are actions shared with neurotransmitters. In developing neuromuscular synapses, acute application of BDNF and NT-3 potentiate synaptic transmission, an effect mediated by Trk receptors (Lohof *et al.*, 1993). This finding also holds true for mature synapses in the hippocampus (Kang & Schuman, 1995). The mechanism is pre-synaptic (Lohof *et al.*, 1993; Kang & Schuman, 1995) and is blocked by cAMP antagonists (Boulanger & Poo, 1999). Further studies have identified a role for neurotrophins in mediating various forms of synaptic plasticity including long-term potentiation (LTP) (Figurov *et al.*, 1996; McAllister *et al.*, 1999). Further acute actions of neurotrophins include the direct modulation of various ion channels and the release of glutamate (Levine *et al.*, 1998; Takei *et al.*, 1998; Yang *et al.*, 2001). There thus appear to be two acute mechanisms of action of neurotrophins: (1) the regulation of ion channels and (2) the regulation of synaptic transmission. Both electrical activity and neurotrophins themselves regulate the synthesis and secretion of neurotrophins (Gall & Isackson, 1989; Funakoshi *et al.*, 1995; Wang & Poo, 1997; Kruttgen *et al.*, 1998). BDNF can be released from newborn rat hippocampal neurons by electrical stimulation and, like neurotransmitter release, is dependent upon the entry of extracellular Ca^{2+} through N-type

voltage gated Ca^{2+} channel (VGCC) (Balkowiec & Katz, 2002). Release may therefore be activity-dependent. Receptors for neurotrophins are also rapidly recruited to the plasma membrane when it is depolarized (Meyer-Franke *et al.*, 1998). Acute focal application of low concentrations of BDNF also elicits action potential generation in hippocampal, cortex and cerebellar neurons, with an onset time similar to that of glutamate (Kafitz *et al.*, 1999). As neurotrophins are released in an activity-dependent manner, regulate ion channels and synaptic transmission, and can rapidly evoke action potential generation in a similar time course to classical neurotransmitters, evidence indicates that neurotrophins act in some systems in a manner analogous to neurotransmitters.

Trophic Actions of GPCR Agonists and Neurotransmitters

As GPCR and neurotrophin receptors can activate similar pathways, it is not surprising that GPCR-coupled agonists can also regulate neuronal growth, survival and differentiation. Pituitary adenylate cyclase-activating polypeptide (PACAP) is perhaps one of the better known GPCR agonists that can function in a neurotrophic manner. PACAP induces neurite outgrowth and differentiation of PC12 cells, a process which is dependent upon ERK, but is independent of Ras (Barrie *et al.*, 1997; Lazarovici *et al.*, 1998). In sympathetic neurons, PACAP as well as vasoactive intestival peptide (VIP), regulates the growth of dendrites, alters the expression of neuropeptide Y (NPY), and up-regulates the levels of tyrosine hydroxylase, leading to increased noradrenaline synthesis (McKeon & Zigmond, 1993; May & Braas, 1995; Mohney & Zigmond, 1998; Drahushuk *et al.*, 2002). Lysophosphatidic acid also activates a GPCR to regulate neuronal differentiation and proliferation. It also functions as either a pro- or anti-apoptotic factor for neuronal cells, depending on the cell type examined and the G-protein activated (Ye *et al.*, 2002).

Recently, neurotrophic roles have been discovered for well known peptide and non-peptide GPCR neurotransmitters. Multiple neurotransmitters have been shown to regulate varying aspects of neuronal maturation (Lauder, 1993; Cameron *et al.*, 1998). The continuous application of the inhibitory transmitter γ-aminobutyric acid (GABA) is known to regulate aspects of neuronal development and differentiation, such as cell

proliferation, dendritic growth, pre-synaptic specialization, and GABA receptor expression, both *in vitro* and *in vivo,* in systems such as SCG neurons and chick cortical and retinal neurons (Wolff *et al.*, 1978; Redburn & Schousboe, 1987; Spoerri, 1988). These effects are mediated by iontrophic $GABA_A$ receptor-induced depolarization in developing neurons, which increases $[Ca^{2+}]_i$ by the activation of VGCC (Yuste & Katz, 1991; Ben Ari, 2002). Depolarization itself is sufficient to support neuronal survival, an effect similar to that produced by neurotrophins. These above effects are most likely due to increases in $[Ca^{2+}]_i$ via increased activity of VGCC, activating immediate early genes, which lead to subsequent activation of transcriptional events (Murphy *et al.*, 1991). GABA is also involved in neuronal migration, as signaling via $GABA_B$ receptors are found to regulate nerve growth cone guidance (Xiang *et al.*, 2002). The transmitter NPY also has been identified as having trophic abilities. NPY acts as a neuroproliferative factor for olfactory neuronal precursor cells, signaling via PKC and ERK (Hansel *et al.*, 2001). As a trophic factor, GnRH exerts its classical effects within the mammalian hypothalamo-hypophyseal system. When released from the hypothalamus GnRH, acting at its GPCR, stimulates the synthesis and release of follicle-stimulating hormone and luteinizing hormone from the anterior pituitary (Cheng & Leung, 2000; Klausen *et al.*, 2002). As discussed above GnRH (LHRH) is also a classical neurotransmitter in BFSG, illustrating how signaling molecules can function as both hormone and neurotransmitters in different systems. Thus in addition to their fast actions over periods of seconds to minutes, neurotransmitters may also function over periods of hours to days with effects similar to those of neurotrophic factors or hormones.

The evidence provided above suggests that neurotransmitters can act like neurotrophic factors and vice versa. Thus, what constitutes a transmitter or a trophic factor may be due to the action of the molecule, and not the nature of the molecule itself.

Trophic Regulation of Na^+ and K^+ Channels

NGF halts the proliferation, and initiates neurite outgrowth and ion channel expression in PC12 cells (Greene & Tischler, 1976). This reflects differentiation of a neuroendocrine cell into a 'sympathetic like' neuron, involving activation of the Ras/ERK and PI3K pathways (Kremer *et al.*, 1991; Pang *et al.*, 1995; Fanger *et al.*,

1997; Boglari *et al.*, 1998). The induction of electrical activity in differentiated PC12 cells upon stimulation with NGF occurs because of the synthesis and expression of ion channels. In addition to inducing channel expression during development of PC12 cells or immature neurons, neurotrophins are also involved in maintaining the expression of ion channels in adult, differentiated neurons. While this thesis examines the regulation of Ca^{2+} channels, it is valuable to summarize the data regarding Na^+ and K^+ channel regulation, as similar mechanisms may be involved in the regulation of all voltage-gated ion channels.

Long-term NGF treatment of PC12 cells (Mandel *et al.*, 1988; Pollock *et al.*, 1990), pancreatic β-cells (Vidaltamayo *et al.*, 2002), astrocytoma cells (Kraft *et al.*, 2001), and BFSG neurons (Lei *et al.*, 2001) induces or increases Na^+ channel expression. Transgenic mice, engineered to over-express NGF have DRG neurons which display increased Na^+ channel expression (Fjell *et al.*, 1999). Long term exposure of both neurons and PC12 cells to a variety of neurotrophins also up-regulates the level of K^+ channels (Garber *et al.*, 1989; Sharma *et al.*, 1993; Holm *et al.*, 1997; Martin-Caraballo & Dryer, 2002; Mizoguchi *et al.*, 2002). Unlike Na^+ channels, K^+ channels in BFSG are unaffected by prolonged NGF treatment (Lei *et al.*, 2001), suggesting that while neurotrophins can regulate neuronal conductances, not all factors behave in an identical manner. This is also true in PC12 cells and murine spiral ganglion neurons. In these cells, different growth factors (activating different receptors) produce different effects on Na^+ and K^+ channels (Fanger *et al.*, 1995; Adamson *et al.*, 2002). This indicates that the specificity of signaling in regards to channel expression is due to differences in the properties of various Trk receptors.

The small G-protein Ras, the main activator of the ERK pathway, has been shown to be non-essential in NGF regulation of Na^+ channels (Fanger *et al.*, 1993), implying that other pathways such as PI3Kγ or SNT may be involved. In BFSG, PI3K inhibition blocks the long term regulation of Na^+ channels in adult BFSG neurons (Lu *et al.*, 2002), suggesting that PI3K may mediate the effects of NGF on Na^+ channels. K^+ channels are differentially regulated by the Ras/Raf/ERK pathway. K_{Ca} channels are up-regulated by the actions of Ras (Huang & Rane, 1994; Pena *et al.*, 2000) and inhibiting Ras blocks the up regulation of K_{Ca} by glial-derived neurotrophic factor (GDNF) (Martin-Caraballo &

Dryer, 2002). However, IRK1 type K^+ channels are downregulated by Ras activation (Giovannardi *et al.*, 2002). Thus in some cases, Ras may be involved in regulating K^+ channel expression, but not in Na^+ channel expression, which may be under the control of other pathways, such as the PI3K pathway.

Trophic Regulation of Ca^{2+} Channels

NGF increases whole-cell Ca^{2+} channel currents in PC12 cells (Streit & Lux, 1987; Furukawa *et al.*, 1993; Lewis *et al.*, 1993; Cavalie *et al.*, 1994). However there are differing reports as to whether NGF only increases the number of channels per unit of cell surface area (current density) (Furukawa *et al.*, 1993; Lewis *et al.*, 1993; Cavalie *et al.*, 1994), or whether the effect of NGF also promotes an increase in the growth of cell neurites and soma thus causing current density to remain unchanged (Streit & Lux, 1987). The effect of NGF is believed to be due to an increase in surface expression of ω-conotoxin GVIA-sensitive N-type Ca^{2+} channels (Streit & Lux, 1987; Lewis *et al.*, 1993; Furukawa *et al.*, 1993). Additionally, both NGF and basic fibroblast growth factor (bFGF) increase the inactivation of whole cell Ca^{2+} currents (Streit & Lux, 1987; Streit & Lux, 1990; Rane & Pollock, 1994).

The effect of NGF is believed, in part, to activate immediate early genes, *c-fos*, and *c-jun*, as NGF increases their mRNA levels and transfection of *c-fos* and *c-jun* cDNA mimicked some of the effects of NGF (Cavalie *et al.*, 1994). The effect of NGF on inducing Ca^{2+} channels in PC12 cells is dependent upon signaling via Ras. However, Ras activation on its own is not sufficient to drive the expression of channels, even though this same activation is sufficient to promote neurite extension (Pollock & Rane, 1996). This suggests that additional activated pathways may be required to drive the expression of Ca^{2+} channels in PC12 cells.

The regulation of Ca^{2+} channels by neurotrophins has also been examined in neurons. In cultures of mouse fetal dorsal root ganglion (DRG) neurons, the long-term exposure to NGF has been found to decrease action potential duration, an effect postulated to be due to modulation of Ca^{2+} channel current (I_{Ca}) (Chalazonitis *et al.*, 1987). In contrast, an *in vivo* study examining the effects of NGF on neonatal rats has shown that action potential duration is increased by the long-term exposure to NGF

(Ritter & Mendell, 1992), suggesting that there may be differing actions of NGF on regulating the action potential *in vivo* compared to *in vitro*. Axotomy results in an increase in action potential width in adult BFSG, which is partially due to a decrease in I_{Ca} (Jassar *et al.*, 1993). The effect of axotomy is similar to the electophysiological changes that occur in explant cultures of BFSG (Traynor *et al.*, 1992). One possible explanation for this finding is that axotomy causes a loss of contact of neurons with their target tissue, which results in a loss of target derived NGF. This supports the hypothesis that NGF is required for the maintenance of Ca^{2+} channels in adult BFSG. Further support for this hypothesis derives from the observation that reversible disconnection of post-synaptic BFSG neurons from their targets promotes reversible alterations in Ca^{2+} channel currents (Petrov *et al.*, 2001). In 12d cultures of adult BFSG neurons maintained in a serum-free, low density cell cultures, NGF promotes an increase in I_{Ca} over control cultures, which is due to an increase in N- and L-type Ca^{2+} channels reduction in inactivation of whole cell I_{Ca} (Lei *et al.*, 1997). In control cultures, I_{Ca} does not change significantly, although I_{Ca} density does decrease, presumably because of the increase in cell membrane surface area as the neurons sprout neurites in culture. Treatment of neurons with NGF does not affect I_{Ca} density, suggesting that the presence of NGF functions to promote increased channel synthesis to maintain I_{Ca} in proportion to the increase in cell membrane (Lei *et al.*, 1997). This effect is dependent upon protein synthesis and the Ras/ERK pathway, but not upon PI3K or PLC (Lei *et al.*, 1998). NGF also up-regulates N- and L-type Ca^{2+} channels in basal forebrain neurons while BDNF has been found to have no effect (Levine *et al.*, 1995). In developing hippocampal neurons NGF, BDNF, and NT-3 up-regulate Ca^{2+} channels, with NGF and NT-3 being more effective in regulating L-type channel, and BDNF being more effect in regulating N-, P/Q- and R- type channels (Baldelli *et al.*, 2000). As in BFSG, this process is also dependent upon protein synthesis and the activation of the Ras/MAPK pathway (Baldelli *et al.*, 2000). Chronic application of BDNF increases spontaneous and evoked GABAergic transmission in this system by upregulating Ca^{2+} channels. Thus, suggesting that the increase in Ca^{2+} channels promotes an increase in efficacy of pre-synaptic transmitter release (Baldelli *et al.*, 2002). Microinjection of anti-p21Ras antibodies in DRG neurons reduces basal I_{Ca}. Additionally, microinjection of oncogenic p21-K-Ras (to

up regulate Ras signaling) increases basal I_{Ca}, suggesting that Ras is involved in the tonic regulation of Ca^{2+} channels in DRG neurons (Fitzgerald & Dolphin, 1997). Chronic application of phorbol 12,13-dibutyrate (an activator of PKC) reduces N-type Ca^{2+} channel expression in chick sympathetic neurons (Przywara *et al.*, 1997). This finding suggests that chronic activation of PKC reduces Ca^{2+} channel expression. However, the chronic application of phorbol esters is known to downregulate, not activate, PKC over the long term bringing into question the results of this report.

Ion Channels in Bullfrog Sympathetic Ganglia Neurons

The above sections have described the signal transduction pathways of GPCRs, the trophic regulation of ion channel expression, as well as the advantages of using BFSG neurons as a model system to study these actions. This section will describe the ion channels within BFSG and their regulation. Na^+, Ca^{2+}, and various types of K^+ channels have been well characterized in BFSG (for review see Smith, 1994). Discussion will be limited to M-type K^+, and Ca^{2+} channels, as these channels are the subject of experiments described in this thesis.

M-Channels

The M-conductance/M-current (g_M/I_M) is a slowly activating, non-inactivating, voltage- and time-dependent K^+ conductance/current (Brown & Adams, 1980). The threshold for g_M activation is about -60mV (Adams *et al.*, 1982a) which is close to the resting membrane potential of BFSG cells. Because g_M generates a hyperpolarizing outward K^+ current at depolarized potentials, it may function as a 'physiological voltage-clamp' to resist the influence of depolarizing, and hyperpolarizing stimuli (Adams *et al.*, 1982a). Agonist-induced g_M suppression leads to membrane depolarization, and the suppression of g_M by ACh and LHRH contribute respectively to the generation of slow e.p.s.p. and the late-slow e.p.s.p (Jones, 1985; Jones, 1989; Smith, 1994).

Genetic analysis has revealed a novel family of K^+ channels called KCNQ channels which play a role in the brain, heart and other tissues. Currently five genes (KCNQ1-5) have been identified, all of which encode K^+ channel subunits of the typical 6 transmembrane domain 'shaker like' K^+ superfamily, ranging in length from 676

(KCNQ1) to ~900 (KCNQ5) amino acids (Robbins, 2001). When heterologously expressed, all members of the KCNQ family can generate M-like currents (Selyanko *et al.*, 2000; Lerche *et al.*, 2000; Schroeder *et al.*, 2000). While KCNQ1 has a high expression in the heart and the inner ear, and KCNQ4 is restricted to the central auditory system and the inner ear (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996; Kubisch *et al.*, 1999), KCNQ2, KCNQ3, and KCNQ5 are widely distributed though out the nervous system (Wang *et al.*, 1998; Cooper *et al.*, 2000; Lerche *et al.*, 2000; Schroeder *et al.*, 2000). In many areas of the nervous system, KCNQ2 and KCNQ3 are co-expressed (Schroeder *et al.*, 1998). Heteromeric expression of KCNQ2 and KCNQ3 channel subunits (KCNQ2/3) is believed to constitute native M-channels in sympathetic ganglia (Wang *et al.*, 1998; Hadley *et al.*, 2000; Selyanko *et al.*, 2000; Shapiro *et al.*, 2000). Heteromeric expression of KCNQ2 and KCNQ3 increases the K^+ current amplitude by a factor of 10 over the expression of either of the subunits as homomers which is due to increased surface expression of the channels (Wang *et al.*, 1998; Yang *et al.*, 1998; Schwake *et al.*, 2000; Selyanko *et al.*, 2001). KCNQ5 is also capable of forming heteromultimers with KCNQ3 which results in a M-like current (Jentsch, 2000; Robbins, 2001) Recent work has suggested that KCNQ5 as well as KCNQ2/3 subunits are responsible for the M-current in rat hippocampal neurons (Shah *et al.*, 2002). However at present, KCNQ2/3 channels are believed to best represent the molecular correlate of the M-channel (Roche *et al.*, 2002).

In BFSG, g_M is inhibited by muscarinic agonists (Brown & Adams, 1980; Adams *et al.*, 1982b; Adams *et al.*, 1986; Jones, 1987a; Selyanko *et al.*, 1990a), peptidergic agonists, including LHRH (Adams & Brown, 1982; Adams *et al.*, 1982b; Adams *et al.*, 1983; Jones, 1985; Pfaffinger *et al.*, 1988; Pfaffinger, 1988; Bosma & Hille, 1989; Bley & Tsien, 1990), and nucleotides (Gruol *et al.*, 1981; Tokimasa & Akasu, 1990; Stemkowski *et al.*, 2002). In native superior cervical ganglia (SCG) neurons, as well in expression systems, g_M is also suppressed by a variety of agonists (muscarinic, peptidergic and nucleotidic) (Shapiro *et al.*, 1994; Jones *et al.*, 1995; Cruzblanca *et al.*, 1998; Selyanko *et al.*, 1999; Shapiro *et al.*, 2000; Selyanko *et al.*, 2000; Haley *et al.*, 2000; Bofill-Cardona *et al.*, 2000; Zhang *et al.*, 2003; Hoshi *et al.*, 2003). Suppression of g_M by these agonists involves a membrane delimited signal, requiring receptor

signalling via pertussis-toxin insensitive $G_{q/11}$ and phospholipase C (PLCβ) (Tokimasa & Akasu, 1990; Selyanko *et al.*, 1992; Caulfield *et al.*, 1994; Jones *et al.*, 1995; Marrion, 1997; Cruzblanca *et al.*, 1998; Haley *et al.*, 1998; Haley *et al.*, 2000; Brown *et al.*, 2000; Guo & Schofield, 2002; Stemkowski *et al.*, 2002; Suh & Hille, 2002).

Although suppression of g_M by muscarinic cholinergic agonists was first described over 20 years ago in BFSG neurons (Brown & Adams, 1980), the transduction processes that couple receptor activation to channel closure are still not fully known. As stated above, receptors that suppress g_M activate PLC which hydrolyzes PIP_2 to generate IP_3 and DAG (Pfaffinger *et al.*, 1988; del Rio *et al.*, 1999; del Rio *et al.*, 1999). Suppression of I_M by bradykinin in SCG neurons, but not in NG108-15 mouse neuroblastoma x rat glioma hybrid cells (Higashida & Brown, 1986; Brown & Higashida, 1988a; Brown & Higashida, 1988b; Cruzblanca *et al.*, 1998) occurs by Ca^{2+} release from IP_3-sensitve Ca^{2+} stores. However this is not the case for other agonists, as their suppression of g_M does not involve hydrolysis products such as PKC, IP_3, or Ca^{2+} downstream of PLC (Pfaffinger *et al.*, 1988; Bosma & Hille, 1989; Selyanko *et al.*, 1990a; Beech *et al.*, 1991; del Rio *et al.*, 1999; Shapiro *et al.*, 2000; Suh & Hille, 2002; Stemkowski *et al.*, 2002). Difficulty in determining the signal transduction mechanisms of agonist induced g_M suppression has stemmed partially from the fact that a variety of different intracellular signaling entities are known to modulate M-channels. Ca^{2+}, DAG, calcinurin, and myosin light chain kinase (MLCK) are all known to modulate g_M (Bosma & Hille, 1989; Selyanko *et al.*, 1990a; Marrion *et al.*, 1991; Akasu *et al.*, 1993; Chen *et al.*, 1994; Marrion, 1994; Marrion, 1996; Selyanko & Brown, 1996). In spite of the overwhelming evidence against PKC in mediating agonist-induced M-channel suppression, this mechanism has been recently been re-examined. Suppression of KCNQ2/3 channels by muscarinic agonists has been shown to involve PKC, by a mechanism dependent upon the multivalent A-kinase-anchoring protein, AKAP150 (Hoshi *et al.*, 2003). However, this effect was only observed after prolonged inhibitor application. Additionally, the effect of blocking PKC prevented only a small proportion of suppression from occurring, suggesting that alternate mechanisms are involved. This suggests that blocking PKC and disrupting the AKAP150/PKC/M-channel complex alters agonist-induced g_M suppression. However, it does not imply that muscarinic agonists

utilize this pathway to suppress M-channels. Thus, it appears that while various second messengers downstream of PLC may modulate g_M, they are not crucial in agonist-induced g_M suppression (Marrion, 1997; Brown & Yu, 2000; Stemkowski *et al.*, 2002). As 'downstream' messengers from PLC activation are not involved in the suppression of M-channels , a novel hypothesis is that g_M suppression may be due to a reduction in membrane phospholipid, PIP_2, which occurs following PLC activation (Willars *et al.*, 1998b). The 'PIP_2 depletion hypothesis' states that M-channels are maintained in an open configuration by the presence of plasma membrane PIP_2, and that agonist stimulation of PLC mediates the hydrolysis of PIP_2 to promote M-channels to assume a closed configuration (i.e. g_M suppression). This constitutes a viable hypothesis to explain the hithero unknown second messenger mediating M-channel suppression. Support for the hypothesis has come from recent work published over the course of this thesis (Suh & Hille, 2002). There is now quite good evidence that agonist-induced PIP_2 depletion may be responsible for muscarine-induced closure of KCNQ2/3 channels in expression systems (Stemkowski *et al.*, 2002; Zhang *et al.*, 2003).

Several other lines of evidence support this hypothesis. Firstly, ATP or metabolic substrates such as glucose or pyruvate are required to prevent M-channel rundown during whole-cell recordings (Pfaffinger, 1988; Tokimasa & Akasu, 1990; Simmons & Schneider, 1998), suggesting that the maintenance of g_M may be dependent upon processes involving the synthesis of PIP_2. Secondly, inhibitors that prevent the recovery from receptor-mediated M-channel suppression also block PIP_2 replenishment at the cell surface suggesting the need for ATP to 'fuel' resynthesis of PIP_2 levels in the membrane to promote I_M recovery (Suh & Hille, 2002; Zhang *et al.*, 2003). This lends support to the idea that the initial M-channel closure may be due to an agonist-induced reduction of PIP_2. Thirdly, direct modulation of KCNQ2/3 channels in an expression system and putative M-channels in SCG neurons by PIP_2 has recently been shown (Zhang *et al.*, 2003). Lastly, a number of cation channels, such as ATP-sensitive K^+ channels (K_{ATP}), G-protein activated inward rectifier K^+ channels (GIRK) (Hilgemann & Ball, 1996; Huang *et al.*, 1998; Baukrowitz *et al.*, 1998; Shyng & Nichols, 1998), HERG K^+ channels (Bian *et al.*, 2001), TRPM7 channels (Runnels *et al.*, 2002) and P/Q- and N-type Ca^{2+} channels (Wu *et al.*, 2002) have been recently found to be modulated by PIP_2

and that the activation of membrane receptors which stimulate PLC-mediated hydrolysis of PIP_2 promotes their inhibition.

The PIP_2 depletion hypothesis has been suggested to explain g_M inhibition by muscarinic agonists in rat sympathetic neurons (Suh & Hille, 2002; Zhang *et al.*, 2003). However, although there is good evidence for its applicability to receptor-mediated inhibition of expressed KCNQ2/3 channels in expression systems (Zhang *et al.*, 2003), unequivocal verification for agonist-induced M-channel suppression in an intact neuron is not yet available

Ca^{2+} Channels

Voltage-gated Ca^{2+} channels (VGCC) are composed of an α_1 subunit, the essential channel moiety, and the auxiliary subunits α_2/δ, β, and γ. The channels are classified according to their α_1 subunits as L- ($Ca_V1.1/\alpha_{1S}$, $Ca_V1.2/\alpha_{1C}$, $Ca_V1.3/\alpha_{1D}$, and $Ca_V1.4/\alpha_{1F}$), N- ($Ca_V2.2/\alpha_{1B}$), P/Q- ($Ca_V2.1/\alpha_{1A}$), R- ($Ca_V2.3/\alpha_{1E}$), and T- ($Ca_V3.1/\alpha_{1G}$, $Ca_V3.2/\alpha_{1H}$, and $Ca_V3.3/\alpha_{1I}$) types (Catterall, 2000). Varying combinations of α_1 subunits with the auxiliary subunits give rise to different combinations of particular types of VGCC (Catterall, 2000). N-, P/Q-, and R-type channels are primarily responsible for Ca^{2+} entry into nerve cells to induce release of neurotransmitters, while L-type channels in the nervous system are primarily responsible for Ca^{2+} entry to cause the initiation of gene transcription and endocrine secretion (Catterall, 2000).

Ca^{2+} channels in BFSG are well characterized (Jones & Marks, 1989a; Jones & Marks, 1989b; Jones & Jacobs, 1990; Sala, 1991; Werz *et al.*, 1993). These channels play an important role in the generation of the action potential in BFSG cells (Pennefather *et al.*, 1985; Smith, 1994). Both activation and deactivation of these channels is rapid when either Ca^{2+} or Ba^{2+} is used as a charge carrier (Jones & Marks, 1989a). While the activation kinetics can be fitted with a gating particle with 3 effective positive charges, better fitting has been reported with two gating particles in a 'm^2' model (Sala, 1991). Kinetics for inactivation are slower and more complex than those for activation/deactivation. Three components have been observed: 'slow' (τ=several minutes), 'intermediate' (τ=150ms), and 'fast' (τ=120ms) (Jones & Marks, 1989b; Werz *et al.*, 1993; Jassar *et al.*, 1993). In acutely dissociated B-neurons ninety percent of the

total Ca^{2+} current is carried by ω-conotoxin GVIA-sensitive N-type channels. Nifedipine has little effect on the total current, suggesting that there are limited numbers of L-type channels in acutely dissociated cells (Jones & Marks, 1989a).

N-type channels in the nervous system are primarily acutely regulated by direct interaction with G-proteins and SNARE proteins, and secondarily by protein phosphorylation (Catterall, 2000). Noradrenaline (NA), ATP, LHRH, substance P, and NPY all inhibit Ca^{2+} currents in BFSG (Schofield & Ikeda, 1988; Jones & Marks, 1989a; Bley & Tsien, 1990; Elmslie *et al.*, 1990; Elmslie, 1992). Inhibition of N-type channels by these GPCR-coupled agonists most likely occurs in a voltage dependent manner by direct interaction of the βγ subunits of G-proteins with the Ca^{2+} channel (Ikeda, 1996; Herlitze *et al.*, 1996). Fewer studies have examined the acute regulation of L-type channels in the nervous system, as modulation of L-channels is primarily known to occur in skeletal and cardiac muscle, via second-messenger activated protein phosphorylation pathways (Catterall, 2000)

Rationale

The previous sections have established similarities in the actions of neurotransmitters and neurotrophic factors. Numerous studies have examined the acute regulation of ion channels by neurotransmitters and the tropic and acute regulation of channels by neurotrophins. In BFSG, NGF regulates the expression of Ca^{2+} channels (Lei *et al.*, 1997). This process is dependent upon activation of the Ras/ERK pathway (Lei *et al.*, 1998). Present within BFSG is the peptide transmitter LHRH. This peptide is known to activate the Ras/ERK pathway in mammalian gonadotropes and in GH_3 cells transfected with rat GnRH-receptor (Sim *et al.*, 1995; Sundaresan *et al.*, 1996; Reiss *et al.*, 1997; Han & Conn, 1999). While the signal transduction pathway of the LHRH-R is not fully known in BFSG, various pathways exist whereby GPCRs lead to activation of the Ras/ERK pathway. This suggests that in BFSG, LHRH may also activate this pathway, and like NGF, may regulate Ca^{2+} channel expression.

The unique properties of BFSG neurons afford an excellent opportunity to study the ability of a neurotransmitter to function as a trophic factor. As already mentioned, BFSG cells can be maintained in culture for ≥14 days in a serum free media which is free

from the complicating effects of exogenously-applied or endogenous neurotrophins. As BFSG neurons are of only two types, larger B-cells and smaller C-cells, identification of the same neuronal type in culture is relatively simple.

In addition to its possible role in long-term regulation of electrical properties of neurons, LHRH also exerts short-term regulation of M-channels. As BFSG neurons are the classical preparation in which to study M-channel regulation, these cells provide a unique opportunity to study the neurotrophic and neuromodulatory actions of a single neurotransmitter in the same cell type.

Statement of the Problem

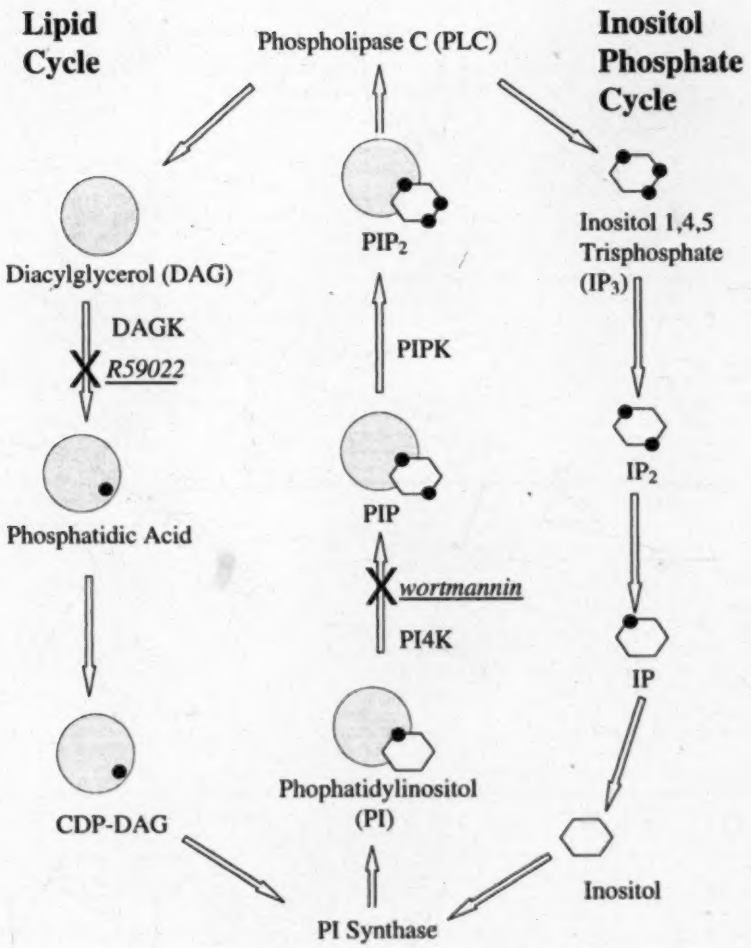
LHRH, ATP, and muscarine suppress M-channels in BFSG but the mechanisms responsible for their actions remain unknown. This thesis first examines the mechanism by which ATP and muscarine suppress g_M. Since there is evidence that not all neurotransmitters suppress g_M by the same mechanism, the thesis next examines whether the peptide transmitter, LHRH, uses the same transduction mechanism as ATP and muscarine to suppress g_M. In view of the emerging concept that neuroransmitters frequently exhibit neurotrophic actions, this thesis will lastly examine if LHRH regulates Ca^{2+} channels in a similar fashion to NGF.

Three hypotheses will therefore be examined in this thesis:

1) That the acute suppression of M-channels by the agonists ATP and muscarine involve depletion of the membrane phospholipid PIP_2.
2) That the acute suppression of M-channels by LHRH similarly involves the depletion of PIP_2.
3) That the neurotransmitter LHRH acts in a manner similar to NGF to regulate the expression of Ca^{2+} channels in the adult nervous system of the frog, and that this effect proceeds via the Ras/ERK pathway.

Figure 1-1. Diagram of the Lipid and Inositol Phosphate Cycles. Large circles represent diacylglycerol, small circles represent inorganic phosphate groups and hexagons represent inositol.

Figure 1-1


Lipid Cycle
Phospholipase C (PLC)
Inositol Phosphate Cycle
PIP2
Inositol 1,4,5 Trisphosphate (IP3)
Diacylglycerol (DAG)
DAGK
R59022
PIPK
IP2
PIP
Phosphatidic Acid
wortmannin
PI4K
IP
Phophatidylinositol (PI)
CDP-DAG
Inositol
PI Synthase

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CHAPTER 2

EXAMINATION OF THE INVOLVEMENT OF DEPLETION OF PHOSPHATIDYLINOSITOL 4,5 BISPHOSPHATE IN MEDIATING THE SUPPRESSION OF M-TYPE K^+ CHANNELS BY ATP AND MUSCARINE

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INTRODUCTION

Suppression of the M-conductance/M-current (g_M/I_M) by neurotransmitters that act via $G_{q/11}$ family of G-proteins produces excitatory effects in a variety of neuron types (Caulfield *et al.*, 1994; Cruzblanca *et al.*, 1998; Haley *et al.*, 1998; Marrion, 1997). Although in SCG neurons bradykinin has been proposed to inhibit g_M via phospholipase C (PLC) and Ca^{2+} release from inositol trisphosphate (IP_3)-sensitive stores (Cruzblanca *et al.*, 1998), this transduction mechanism does not explain the actions of other neurotransmitters hitherto studied (Pfaffinger *et al.*, 1988).(Brown & Higashida, 1988a; Brown & Higashida, 1988b; Marrion, 1997; Selyanko *et al.*, 1990; Stemkowski *et al.*, 2002; Suh & Hille, 2002). As discussed in the *General Indroduction,* since 'downstream' messengers are not involved in the actions of most neurotransmitters, it is hypothesized that 'upstream' signaling from PLC, which involves a depletion of the membrane phospholipid, PIP_2 mediates g_M suppression by other agonists. In this model, 'the PIP_2 depletion hypothesis', endogenous PIP_2 would maintain M-channels in the open state. PIP_2 depletion by PLC hydrolysis upon agonist stimulation would promote M-channel closure.

ATP and/or UTP interact with P2Y receptors to suppress g_M and to excite sympathetic neurons (Adams *et al.*, 1982; Bofill-Cardona *et al.*, 2000). A recent study in amphibian neurons has confirmed that, as for other agonists, this response involves PLC but none of the usual downstream second messengers; *i.e.* not Ca^{2+}, IP_3 or PKC (Stemkowski *et al.*, 2002 see also Pfaffinger *et al.*, 1988; Bofill-Cardona *et al.*, 2000). In the present work, it is shown that ATP- and muscarine-induced g_M suppression depends on the operation of the inositol phosphate and lipid cycles. Furthermore, when a g_M-like conductance is expressed in tsA 201 or COS-1 cells following their transfection with the KCNQ2/3 gene (Wang *et al.*, 1998), current 'rundown' is slowed by the inclusion of PIP_2 in the recording pipette. These and other data support the suggestion that the membrane phospholipid PIP_2, favors an open state of the M-channel and that g_M suppression occurs by PLC-mediated PIP_2 hydrolysis (Stemkowski *et al.*, 2002; Suh & Hille, 2002; Zhang *et al.*, 2003).

Methods

Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and experimental protocols were approved by the Health Sciences Animal Welfare Committee of the University of Alberta.

BFSG Experiments.

Neurones in the VIIIth to Xth paravertebral sympathetic ganglia of male and female *Rana catesbeiana* were dissociated using trypsin (Sigma) and type 1A collagenase (Sigma) for 42-45 min, at 37 °C as previously described (Kurenny *et al.*, 1994; Selyanko *et al.*, 1990). Final dissociation was accomplished by trituration with a 1ml Pasteur pipette. The dissociated cells were then redistributed into 2.5 ml of medium in each of 20 35-mm polystyrene tissue culture dishes (Nunc). Experiments were done on neurones that were maintained for 1-2 days in a culture medium which consisted of diluted L-15 medium (73%) supplemented with 10 mM glucose, 1 mM $CaCl_2$, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 μM cytosine arabinoside. Electrophysiological recordings were carried out at ~ 20 °C using whole-cell recordings (Stemkowski *et al.*, 2002) (Axoclamp 1B amplifier, pClamp 5.5. software). Resting membrane potential was -50 to -55 mV and cells were held at -30 mV. Experiments with agonists (ATP, and muscarine) were carried out on B-cells (C_{in} > 30 pF) whereas PIP_2 was introduced into the smaller C-cells (C_{in} < 25 pF) (Kurenny *et al.*, 1994). Since the currents to be recorded were usually <0.5 nA, no corrections were made for the voltage-drop across the series resistance (Selyanko *et al.*, 1990), Current-voltage relationships were obtained using a 4.5 s ramp command from the holding potential of -30 mV to -110 mV (~18mV.s^{-1}). Leak current (I_L) at -30 mV was estimated by extrapolation of the I-V plot obtained at voltages between –75 and -90 mV. The percentage of agonist-induced g_M suppression was calculated from the formula:

$$\% g_M \text{ suppression at -30 mV} = 1 - \frac{(I_M \text{ at -30 mV in presence of agonist}) - (\text{extrapolated } I_L \text{ at -30 mV})}{(\text{total } I_M \text{ at -30 mV}) - (\text{extrapolated } I_L \text{ at -30 mV})}$$

Extracellular solution contained (in mM), NaCl 113, KCl 6, $MgCl_2$ 2, $CaCl_2$ 2, HEPES/NaOH (pH 7.2) 5 and D-glucose 10. Patch pipettes had d.c. resistances of 3-10 MΩ. Pipette solution contained (in mM), KCl 110, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 0.4, EGTA 4.4, HEPES/KOH (pH 7.0) 5, D-glucose 10, Na_2ATP 2. (pCa = 7).

Experiments on tsA 201 and COS-1 cells

tsA-201 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10% fetal calf serum and 0.1% penicillin/streptomycin at 37 °c with 10% CO_2. KCNQ2 and KCNQ3 channel subunit clones were kindly provided by Dr. D. McKinnon. Cells were plated at ~40-70% confluency on 35 mm dishes 4-6 hrs prior to transfection. Clones were inserted into the mammalian expression vector pCDNA3 and transfected into COS-1 cells using Lipofectamine reagent as per manufacturer's instructions (Life Technologies) or into tsA-201 cells using the calcium phosphate precipitation technique. For expression of heteromultimers, 1:1 molar ratios of KCNQ2 and KCNQ3 cDNA were transfected. Successfully transfected cells were identified by co-expression of the green fluorescent protein plasmid (pGreen-Lantern, Life Technologies), visualized using fluorescence optics.

In whole-cell mode with 2-6MΩ electrodes, recordings were made from COS-1 or tsA-201 cells, 48-72 h or 72-96 h respectively after transfecting. Data were acquired using pClamp software (version 8.0), using an Axopatch 200B amplifier (Axon Instruments) and filtered at 2 kHz. Capacitance transients were cancelled using circuitry within the amplifier and the series resistance compensation was set at 40-60%. Recordings from transfected cells were made at ~ 20 °C using an extracellular solution consisting of (in mM) NaCl 144, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 0.5, HEPES 5, D-glucose 10 (pH 7.4), and an intracellular solution consisting of (in mM) K-acetate 80, KCl 30, HEPES 40, $MgCl_2$ 3, EGTA 3, $CaCl_2$ 1 (pH 7.4). The K_{ATP} channel Kir6.2 subunit clone from mouse was generously provided by Dr S. Seino and the SUR1 subunit clone from hamster was generously provided by Drs. L. Aguilar Bryan and J. Bryan. Clones were inserted into the mammalian expression vector pCNDA3. K_{ATP} channels were transfected as 1:1 molar ratios of Kir6.2 and SUR1 into tsA-201 cells with the green

fluorescent protein plasmid in an analogous manner to KCNQ2/3 channels. K_{ATP} channels were recorded under symmetrical K^+ conditions with a pipette solution and superfusion system containing (in mM) KCl 110, KOH 30, HEPES 5, $MgCl_2$ 1, EGTA 10 (pH 7.4) in an inside-out patch configuration. Membrane patches were held at -60 mV and were directly exposed to test MgATP solutions via a multi-input perfusion pipette (time required to change solution at the tip of the recording pipette was less than 2 sec). All experiments were performed at room temperature (20-22 °C).

Drugs and Chemicals.

Drugs were applied to BFSG neurones using a rapid superfusion system that was constructed from 0.8mm diameter polyimide tubes (Cole Palmer, Vernon Hills, IL). These were connected via a tap system to a series of reservoirs, and their tips fed into a small-volume mixing chamber. The mixing chamber fed into another 0.8 mm polymide tube that was placed near the neuron under study. Control ramps were applied to measure I_M while extracellular solution was applied from the superfusion system, the flow was then switched to allow superfusion of drugs and a second voltage command applied. Aliquots of U73122, (1-(6-((17β-3methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione, U-73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-pyrrole-2,5-dione, or R59022 (6-(2-[4-([4-fluorophenyl] phenymethylene)-1-piperidinyl]ethyl)-7-methyl-5H-thiazolo(3,2-a) pyrimidin-5-one) were first dissolved in chloroform which was then allowed to evaporate under a stream of nitrogen to yield a filmy residue which was stored at –20 °C until the day of the experiment when it was dissolved fresh in DMSO. LY294002 ((4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), ML-7 (1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine) hydrochloride, and wortmannin were also dissolved in fresh DMSO. Serial dilutions of drugs in external solution were arranged so that the final concentration of DMSO in solutions applied to cells was <0.1 %. Acute application of 0.1 % DMSO had no noticeable effect on their electrophysiological properties (see table 1). PIP_2 was dissolved in chloroform and aliquots stored at –20 °C under N_2. On the day of the experiment, the chloroform was evaporated with a stream of N_2 to leave a filmy

residue of PIP_2. Recording solution was mixed with this residue for about 10min prior to sonnication on ice until the solution was clear (about 30 min).

U73122, U73343, ML-7, R59022 and wortmannin were from Biomol (Plymouth Meeting, PA, USA), PIP_2 was from Calbiochem (San Diego, CA, USA), LY294002 was from Alomone (Jerusalem, Isreal) and PIP_2 Antiserum (immunogen: PIP_2 from bovine spinal cord, animal immunized: mouse) was from Assay Designs Inc. (Anne Arbor, MI, USA). All other chemicals were from Sigma (Oakville, ON, Canada). Data are expressed as mean ± S.E.M. and significance of difference estimated using Student's two tailed *t*-test. Paired tests were used for pairs of observations on the same cell and unpaired tests were used for comparison of data from groups of cells. Data were considered significantly different when $p<0.05$.

RESULTS

Inhibition of PIP_2 re-synthesis slows recovery from g_M suppression

Wortmannin is a non-selective kinase inhibitor that inhibits myosin light chain kinase and phosphatidylinositol 3 kinase (PI3K) (IC_{50} = 50nM in cell culture assays) (Yano *et al.*, 1993). The latter is involved in the synthesis of phosphatidylinositol 3,4,5 trisphosphate (PIP_3) from PIP_2. At relatively high concentrations, wortmannin also inhibits phosphatidylinositol 4 kinase (PI4K) (Willars *et al.*, 1998; Xie *et al.*, 1999b). This enzyme plays a pivotal role in the lipid and inositol phosphate cycles as it is involved in the synthesis of phosphatidylinositol 4 phosphate (PIP) from phosphatidyl inositol (PI). PIP is phosphorylated to PIP_2 by phosphatidylinositol-4-phosphate-5- kinase (PIPK; see Fig 1-1). If PLC-induced PIP_2 depletion is required for agonist-induced g_M suppression, the recovery of the response would require PIP_2 re-synthesis. This recovery should be slowed in the presence of wortmannin because it would impair PIP_2 re-synthesis by its action on PI4K (Runnels *et al.*, 2002; Suh & Hille, 2002) and reduction in the steady-state level of PIP. The data illustrated in Fig 2-1A, which are whole-cell recordings from a BFSG B-neuron, are consistent with this prediction. The traces in Fig 2-1A, which are chart recordings of steady-state I_M at –30 mV, are interrupted by responses to voltage ramps that were used to monitor agonist-induced changes in membrane conductance. These ramps produce I-V plots such as those shown in Fig 2-2

(Selyanko *et al.*, 1990). The I-V plot shown in Fig 2-2 illustrates that the effect of the extracellularly-applied P2Y agonist, ATP is confined to the g_M activation range. The absence of any conductance change at potentials more negative than –70 mV excludes the participation of P2X receptors in the response to ATP. Prior to the application of 10 μM wortmannin, extracellular application of 250 μM ATP produces pronounced g_M suppression (Fig 2-1A). This is reflected as reduction in steady-state outward current in the g_M activation range (positive to –70 mV, see Fig 2-2). Steady-state I_M/g_M returns to its control value within 1-2 min of initiating ATP wash out (white arrow, Fig 2-1A). After 5 min in 10 μM wortmannin, the recovery phase of the ATP response is slowed so that the response is almost irreversible (black arrow, Fig 2-1A) and persistent suppression of g_M is seen. The time for 50 % recovery of ATP responses increased by ten fold, from 19.7±2.5 s to 198.1±33.5 s ($n=11$; $p<0.0005$) in neurons studied with whole-cell recording. The effects of wortmannin are summarized in Fig 2-3. No significant slowing of rate of recovery of successive ATP responses was seen in neurons studied in the absence of wortmannin with whole-cell methods (Fig 2-3). Figure 2-1B shows that wortmannin had little effect on steady-state g_M when it was applied alone for the 10 min time period of the experiment. Wortmannin thus produces an 'agonist dependent' block of g_M.

If the effect of wortmannin reflected an action of PI3K rather than PI4K, a PI3K inhibitor, such as LY294002 (IC_{50} = 1.4 μM in isolated enzyme assays), should affect ATP responses in a similar fashion to wortmannin. This was not the case. Although LY294002 (10 μM) slightly slowed the recovery of ATP responses studied with whole-cell recording (Fig 2-1C), this effect was not significant compared to the time-matched control group (Fig 2-3). Moreover, no slowing of recovery of ATP responses was seen when the effects of LY294002 were studied using perforated patches (Fig 2-3). LY294002 thus did not produce an 'agonist-dependent' block of steady-state g_M (Fig 2-1C).

To show that the effect of wortmannin did not reflect its action on myosin light chain kinase, we tested whether the inhibitor ML-7 would slow recovery of ATP responses in a similar fashion to wortmannin. ML-7 (10 μM) produced a rapidly developing and rapidly reversible reduction in g_M that was associated with an increase in

membrane noise (grey arrow, Fig 2-1D). Although this is suggestive of a channel block mechanism, the effect of ML-7 on g_M did not exhibit any obvious voltage- or use-dependence (unpublished observations). It may therefore reflect a *bona fide* action on myosin light chain kinase; an enzyme that has been reported to affect g_M (Akasu *et al.*, 1993; Tokimasa *et al.*, 1995). Despite this, ATP still suppressed the g_M that persisted in the presence of ML-7 and, more importantly, the time course of recovery of the response was little changed (Fig 2-1D and Fig 2-3). The pronounced slowing of recovery seen with wortmannin (Fig 2-1A) thus cannot be attributed to an action on myosin light chain kinase.

Inhibition of the lipid cycle slows recovery from g_M suppression

The above observations on ATP responses are similar to those of Suh and Hille (2002) who suggested that re-synthesis of PIP_2 is required for recovery of muscarine-induced g_M suppression in rat superior cervical ganglion neurons and for the recovery of muscarinic suppression of KCNQ2/3 channel currents expressed in tsA-201 cells. If this is so, depletion of phosphatidylinositol (PI), which is the substrate for PI4K (Fig 1-1), would also be expected to slow g_M recovery. This is because, by classical enzyme kinetics, the rate of synthesis of phosphatidylinositol phosphates by PI4K will depend on the concentration of the substrate, PI. We therefore interrupted the lipid cycle after DAG using the DAG kinase inhibitor, R59022 (IC_{50} = 10 μM in cell culture assays) (40 μM). A typical experiment is illustrated in Fig 2-4A. The initial 5min exposure to R59022 had little effect on the rate of recovery of the ATP response. However, a second test of ATP in the continued presence of R59022 produced a response with slowed recovery. This trend (progressive slowing of recovery of successive ATP responses in the continued presence of R59022) is apparent in the summarized data for all cells tested (Fig 2-4B). Very little slowing of the recovery of successive ATP responses was seen in control neurons. Thus, the time for 50 % recovery of the ATP response in untreated neurons was 32.6±5.1 s (n=8) and this was significantly less than the 56.7±8.1 s (n=6) seen in neurons after 10 min in 40 μM R59022 ($p<0.025$). We suggest that after one test of ATP in the presence of R59022, the membrane concentrations of PI and PIP are still sufficient to sustain the normal rate of resynthesis of PIP_2 via PI4K and PIPK (see Fig 1-1). M-

channels thus reopen at the control rate. With the second application of ATP however, PI and PIP may be irreversibly depleted because their re-synthesis has been interrupted by R59022 inhibition of DAG kinase (Fig 1-1). The corresponding rate of g_M recovery is thus reduced. These findings are consistent with a role for PIP_2 re-synthesis in g_M recovery (Suh & Hille, 2002), and further implicate earlier intermediates of the lipid cycle as the source of phosphatidic acid for this effect (see Fig 1-1).

PIP_2 Antibodies Attenuate ATP-Induced g_M Suppression

Studies of PIP_2 modulation of other types of K^+ channels (Huang *et al.*, 1998; Xie *et al.*, 1999a; Bian *et al.*, 2001) has led to the development of well-characterized PIP_2 'neutralizing antibodies' that reduce PIP_2 dependent channel activity in a variety of cell types (Huang *et al.*, 1998; Liou *et al.*, 1999; Bian *et al.*, 2001; Chuang *et al.*, 2001). Under the conditions of our experiments, inclusion of PIP_2 neutralizing antiserum (1:100) in the patch pipette failed to affect *resting* g_M in BFSG neurons (Fig 2-5A). Despite this, the *modulation* of g_M by ATP was consistently disrupted (Fig 2-5A). Thus, after 5 min recording with antibody in the pipette, 250 μM ATP suppressed g_M by 85.6±3.1 % but after 25 min of recording, the agonist effectiveness was reduced so that only 42.9±15.2 % suppression was seen (n=4, p=0.05, paired t-test). The steady state-g_M was little changed by the antibody (gray arrows, Fig 2-5A) whereas the amount of ATP-induced g_M suppression was far greater for the first response (white arrow, Fig 2-5A) than for the second response (black arrow, Fig 2-5A). The effect of antiserum was not seen in control experiments where an appropriate amount of horse serum (to control for the excess protein applied to neurons) was included in the recording pipette (Fig 2-5B). Thus, ATP suppressed g_M by 83.4±8.0 % after 5 min with the intracellular horse serum and by 76.1±7.9 % after 25 min (n=5 p>0.5). Fig 2-6A and 2-6B illustrate summarized data of the effect of PIP_2-antibodies and horse serum on steady-state g_M and ATP-induced g_M suppression. One interpretation of these findings is that the antibody preferentially inhibits the PIP_2-PLC interaction rather than a PIP_2-M-channel interaction.

Direct Effects of PIP_2 on KCNQ2/3 Channels

If M-channel closure is promoted by the PLC-mediated hydrolysis of PIP_2 (Suh & Hille, 2002), steady-state g_M should be increased in the presence of exogenously-applied PIP_2 (Ikeda & Kammermeier, 2002). One recent report suggests that exogenously-applied PIP_2 increases putative M-current activity in cell-attached excised patches from rat sympathetic neurons (Zhang *et al.*, 2003). This seemingly simple experiment is remarkably difficult to carry out. One issue may be the simple physical problem of taking a hydrophobic lipid, such as PIP_2 and delivering to the cell membrane *via* the aqueous environment of the recording solutions. We therefore first sought to verify the effectiveness of our aqueous solutions of PIP_2.

Because PIP_2 is known to decrease the sensitivity of K_{ATP} channels to blockade by intracellular ATP (Baukrowitz *et al.*, 1998; Fan & Makielski, 1997) we used this effect to confirm the activity of our PIP_2 solutions. K_{ATP} channels were expressed in tsA-201 cells and PIP_2 was included in the recording pipette on the extracellular side of excised, inside-out patches (see inset to Fig 2-7A). PIP_2 is able to reach the cytosolic face to modulate K_{ATP} channels when applied to the extracellular face (Wada *et al.*, 2002). After 5 min, control channels displayed similar ATP sensitivity to that initially recorded (n=2, Fig 2-7A). Channels exposed to PIP_2 (20 μM), exhibited a marked reduction in sensitivity to intracellular ATP, as substantially greater current was available at 0.1 mM ATP, and even at 1 mM (a concentration which fully inhibited control channels) (n=2, Fig 2-7B).

Since the majority of the studies demonstrating effects of PIP_2 on K^+ channels have been carried out on expressed (Bian *et al.*, 2001; Huang *et al.*, 1998; Zhang *et al.*, 1999) rather than on native channels, we next used whole-cell recording to examine the effect of PIP_2 on KCNQ2/3 channels expressed in tsA-201 or COS-1 cells (Wang *et al.*, 1998). In cells where the channels were successfully transfected, voltage commands from –20 to –50 mV produced a classical g_M relaxation (Brown & Adams, 1980) in response to the start of the voltage pulse and reactivation of the conductance when the membrane voltage was restored to –20 mV (Fig 2-8A; inset). Under the conditions of our experiments, KCNQ2/3 channel currents in tsA-201 or COS-1 cells displayed significant 'run down' during the initial 5 min of recording with ATP-free internal solution in the

pipette. The presence of ATP in the intracellular pipette, or metabolic substrates such as pyruvate or glucose in the extracellular solution, are known to slow the rate of rundown of M-channels during whole-cell recording (Pfaffinger, 1988; Simmons & Schneider, 1998; Suh & Hille, 2002). This suggests that ATP may is required for lipid kinase activity (i.e. PI4K, PIPK) and thus for the maintenance of PIP_2 levels in the membrane (Suh & Hille, 2002). If PIP_2 is required to maintain M-channel function, application of this lipid via the patch pipette would therefore be expected to slow rundown. Fig 2-8A compares the time course of change of KCNQ2/3 channel current, measured from tail currents at –50 mV, in 16 control cells with that in 11 cells recorded with 20 μM PIP_2 in the recording pipette. In control cells, the current amplitude decreased to 45.2±8.8% of initial (n=16) after 3min of recording. By contrast, KCNQ2/3 channel current recorded with 20 μM PIP_2 in the recording pipette decreased significantly less, to 83.3±16.6 % of initial (n=11, $P<0.05$). In other experiments, the I-V characteristics of expressed KCNQ2/3 channels were studied over a wide range using 4.75s commands from -80 to +50 mV. In these experiments, current recorded at +50mV decreased to 58.6±6.2 % of control (n=27) after 5min using normal intracellular solution. Inclusion of 20 μM PIP_2 in the pipette significantly reduced 'run down' of these currents such that those recorded after 5 min were still 85.1±10.8 % of control (n=12, $P<0.05$). Typical experiments are illustrated in Figs 2-8B and 2-8C. Thus, 20 μM PIP_2 is able to modulate KCNQ2/3 channels expressed in tsA-201 or COS-1 cells. This concentration was chosen as it is within the 8-50 μM range of concentrations used in other investigations of phospholipid modulation of ion channels in other systems (Bian *et al.*, 2001; Runnels *et al.*, 2002; Zhang *et al.*, 2003).

Effect of PIP_2 on M-currents in BFSG cells.

To study the effect of PIP_2 on native neurons, we examined its effect on the smaller C-cells of BFSG that express an agonist-sensitive g_M (Jones, 1987), similar to that of the larger B-cells that were used above. We postulated that the smaller volume of the C-cell cytoplasm (Dodd & Horn, 1983; Kurenny *et al.*, 1994) would favor the partition of PIP_2 into their membranes. As with the experiments on KCNQ2/3 channels in COS-1 or tsA-201 cells, ATP was excluded from the intracellular solution when PIP_2 was

tested on BFSG neurons. Although 20 μM PIP_2 slowed run down of KCNQ2/3 currents, this concentration failed to affect g_M in BFSG C-cells. Fig 2-10 shows that there was no significant change in g_M in these cells between 30s and 4min of recording either with or without 20 μM PIP_2 in the recording pipette (Student's paired T-test). Moreover, there was no significant difference in the amount of g_M in the two groups of cells after 4 min of recording (Student's unpaired T-test).

By contrast, the amplitude of g_M recorded following the intracellular application of a high concentration of PIP_2 (100μM) was significantly larger after 4 min recording than after 30 s of recording ($P<0.02$, using Student's paired t-tests on data from each cell to compare current values at 30 s and 4 min; Fig 2-9B and C). This effect of PIP_2 is rather modest because there was no significant difference in the amount of g_M in control cell population compared to the 100 μM PIP_2-treated population after 4 min of recording (Student's unpaired t-test was used to compare all control current values with all values measured in the presence of PIP_2 at 4 min; Fig 2-9A). Figure 2-9A compares the recorded values of g_M over the first 5 min of recording in cells with and without 100 μM PIP_2. Although significant differences are seen at early time intervals, the effect of PIP_2 on g_M in BFSG is very small compared to its effect on KCNQ2/3 channels (Fig 8A).

Intracellular application of 100 μM PIP_2 also did not interfere with the ability of P2Y agonists to suppress g_M. Thus, extracellular application of 250 μM ATP suppressed g_M by 66.9±6.2 % (n=11) in PIP_2 treated cells and this was not different from the amount of suppression seen in control cells 61.5±9.2 % (n=9) ($P>0.6$). This lack of effect on agonist-induced responses may have reflected our inability to alter PIP_2 concentration in the vicinity of the channels, had this occurred, the amplitude of the steady-state current may have been more convincingly increased (see Fig 2-9A).

Investigation of limited effectiveness of PIP_2 on BFSG neurons.

The limited effectiveness of PIP_2 in BFSG neurons could be due to degradation of exogenously-applied PIP_2 by endogenous lipid phosphatases (Zhang *et al.*, 1999). To test this, we inhibited lipid phosphatases using an internal solution containing sodium fluoride (5 mM), sodium orthovanadate (0.1 mM) and sodium pyrophosphate (10 mM) (FVPP

solution) (Huang *et al.*, 1998). Inclusion of a FVPP solution in the recording pipette however did not increase the effectiveness of PIP_2 (Fig 2-10).

Alternatively, the limited effectiveness of exogenously applied PIP_2 may reflect saturation of binding sites on M-channels by endogenous PIP_2. In an attempt to deplete endogenous PIP_2, we applied (extracellular) ATP to activate P2Y receptors and to promote PLC-induced PIP_2 hydrolysis. This was done in the presence of wortmannin to prevent PIP_2 resynthesis prior to gaining whole-cell access to the cell. Cells were thus pretreated with 10 μM wortmannin for 5 min after which ATP was applied for 20 sec. At this time, whole-cell access was obtained and the g_M recorded after 30 s compared to that recorded after 4 min. When cells were treated in this manner, inclusion of PIP_2 (20 μM) in the recording pipette did not alter g_M (Fig 2-10).

PIP_2 (20 μM) was also included in the patch pipette after PLC had been inhibited with U73122 (5 min; 10 μM) to determine if tonically-active PLC was hydrolyzing the applied PIP_2. Under these conditions however, PIP_2 still failed to increase in g_M (Fig 2-10).

Thus, the poor efficacy of exogenously-applied PIP_2 in modulating g_M under the conditions of our experiments is unlikely to reflect the presence of endogenous phosphatases (Huang *et al.*, 1998), saturation of possible binding sites on M-channels by endogenous PIP_2 or ongoing catabolism of PIP_2 by the action of PLC.

Effects of Al^{3+}

Because negative charges on the phosphate moieties of phosphatidylinositides are involved in their binding to positively charged residues on K^+ channels (Zhang *et al.*, 1999), trivalent cations such as Al^{3+} strongly disrupt PIP_2 - channel interactions (Hilgemann & Ball, 1996). To test whether Al^{3+} affected M-channels, KCNQ2/3 channels were studied in inside-out macropatches excised from tsA-201 cells. Apart from leak, almost all current in such patches should be due to KCNQ2/3 current. We found that the total current at 0mV was suppressed by 47.0±6.7 % by 50μM Al^{3+} (n=5). In the experiment is illustrated in Fig 2-11, addition of 1mM ATP, to the intracellular surface of the membrane so as to promote PIP_2 synthesis (Suh & Hille, 2002), partly reversed the effect of Al^{3+}.

PIP_2 and Suppression of g_M by Muscarine.

The M-current was first defined as a muscarine-sensitive current in BFSG neurons (Brown & Adams, 1980). Suppression of g_M by muscarinic agonists in rat superior cervical ganglion neurons is attenuated by 3 μM U73122 and this had led to the conclusion that PLC likely participates in the transduction mechanism for this agonist in mammalian sympathetic ganglia (Suh & Hille, 2002). Although the PLC inhibitor, U73122 (10 μM) antagonized the effect of 10 μM muscarine on g_M in BFSG B-neurons (Fig 2-12A; Table 2-1), this effect was also seen with the *inactive* isomer, U73343 (Fig 2-12B; Table 2-1). The amount of muscarine-induced g_M suppression seen in the presence of U73122 thus did not differ from the amount of suppression seen in the presence of the inactive isomer ($P = 0.5$, Fig 2-13; Table 2-1). Although at 3 μM, no difference was seen with U73122 compared to the inactive isomer, ($P<0.05$, Figure 2-13; Table 2-1), a selective effect was seen once the concentration of U73122 and U73343 was lowered to 1μM ($P<0.05$; Fig 2-13). On the basis of these data, it was not possible to unequivocally implicate PLC in the action of muscarine on g_M in BFSG neurons.

Despite this, the muscarine response was affected in much the same way as the ATP response by both wortmannin (Fig 2-14) and the PIP_2 antiserum (Fig 2-15A and B). Thus, wortmannin slowed recovery of muscarine responses. Prior to the application of wortmannin, the time for 50% recovery of responses to 2 μM muscarine was 23.0±3.9 s. This increased to 174±72.9 s after 10 min in wortmannin. After 5 min recording with the PIP_2 antibody in the pipette, 2 μM muscarine suppressed g_M by 87.0±6.3 % (n=5). After 25 min with the antibody, muscarine became significantly less effective as it suppressed g_M by only 64.0±8.7 % (n=5, $p<0.002$). In control experiments, muscarine suppressed g_M by 82.0±4.5 % after 5min with the intracellular horse serum and by 76.6±3.0 % after 25 min (n=6 $p>0.3$). The effect of muscarine and P2Y agonists on g_M may therefore proceed via similar mechanisms

DISCUSSION

The above results show that perturbation of the lipid and inositol phosphate cycles have effects on ATP- and muscarine-induced g_M suppression in BFSG as would be

predicted if the PLC-mediated depletion of PIP_2 is the signal for channel closure (Suh & Hille, 2002). This mechanism, termed the 'lipid kinase and PI-polyphosphate hypothesis' (PIP_2 depletion hyothesis), was suggested to explain g_M inhibition by muscarinic agonists in rat sympathetic neurons (Suh & Hille, 2002; Zhang *et al.*, 2003). Good evidence for the applicability of this hypothesis is available to explain receptor-mediated inhibition of expressed KCNQ2/3 channels (Zhang *et al.*, 2003). However at this time unequivocal verification of the PIP_2 depletion hypothesis for agonist-induced g_M suppression in an intact neuron is not yet available.

Transduction of Agonist-Induced M-Channel Suppression

Although ATP responses are antagonized by the PLC inhibitor, U73122 and not by the inactive isomer, U73343 (Stemkowski *et al.*, 2002), a preferential effect of U73122 on muscarine-induced g_M inhibition was more difficult to demonstrate (Table 2-1; Fig 2-13). Certainly at 1μM, the active isomer was more effective. This suggests that PLC is involved in muscarine-induced g_M suppression in BFSG neurons as has been suggested in rat neurons by Suh and Hille (2002). Because 3 μM, and 10 μM U73343 failed to affect ATP responses (Stemkowski *et al.*, 2002), yet blocked muscarine responses (Fig 2-12B), this inactive isomer may interact with muscarinic receptors. However the possibility that the inactive analogue acts in a similar manner to the active analogue at high doses can not be ruled out at this time. The inhibitory effect of the inactive isomer at higher concentrations, prevents verification that muscarine-induced g_M-suppression involves PLC in BFSG. In rat SCG neurons U73343 at 3 μM also inhibits muscarine-induced g_M suppression, but not to the same extent as in BFSG (compare Suh and Hille 2002; Fig 2C with Fig 2-13). Examination of the effects of 10 μM U73343 on muscarinic responses in SCG neurons would indicate whether this compound interacts with muscarinic receptors in mammalian neurons, as it does in amphibian neurons. This would help to validate that muscarine-induced g_M suppression, in some systems, does involved PLC. With the use of antisense-expression constructs, muscarine, but not bradykinin, induced g_M suppression was proposed to not involve PLC (Haley *et al.*, 2000), illustrating the conflicting results as to the role of PLC in mediating suppression of M-channels by muscarine.

Wortmannin slows recovery of P2Y responses. This effect can be attributed to its action on PI4K as it is not produced by the PI3K inhibitor LY294002 or the myosin light chain kinase inhibitor, ML-7. Similar mechanisms appear to operate in intact and perfused neurones as the effects of wortmannin were seen with both whole-cell and perforated patch recording (data not shown). The inhibition of g_M by wortmannin has been previously described in BFSG and was attributed to inhibition of MLCK (Tokimasa *et al.*, 1995). However from experiments in BFSG reported here, and in tsA-201 cells in the work of Suh and Hille (2002), it appears that the inhibition of g_M by wortmannin reported earlier was misinterpreted and more likely due to its actions on PI4K.

Our findings differ from those of a previous study in which wortmannin had a direct inhibitory effect on g_M in BFSG neurons that was independent of the presence of agonist (Tokimasa *et al.*, 1995). Differences in the nucleotide content of the internal solutions used in the two studies may provide an explanation for this disparity. Whereas our internal solution contained 2 mM ATP and no GTP, that used by Tokimasa et al. (1995) contained less ATP (1.15 mM) and 1.5 mM GTP. Our solution, with the relatively high ATP content would favour PIP_2 synthesis (Suh & Hille, 2002), whereas the presence of GTP in the solution used by Tokimasa et al. (1995), may favour constitutive G-protein turnover, tonic activation of PLC and hence a tendency towards PIP_2 hydrolysis. Addition of wortmannin under these conditions would prevent PIP_2 re-synthesis and this may explain the direct, agonist-independent action of wortmannin on g_M observed Tokimasa et al. (1995). 10 μM wortmannin also affected steady-state g_M in rat sympathetic neurons (Suh & Hille, 2002) to a greater extent than in BFSG. This may reflect species differences. For example, there may be greater pools of PIP_2 available in frog neurons compared to rat neurons. Alternatively, the presence of a low concentration of GTP (0.1 mM) in the pipette solutions used by Suh and Hille (2002) may have favoured ongoing PLC activity.

Inhibition of the lipid cycle at the level of diacylglycerol kinase, by using R59022, also slowed recovery of ATP responses but this effect developed more slowly than that of wortmannin. Prolonged activation of PLC will deplete the membrane of PIP_2 and PIP (Willars *et al.*, 1998). Generation of PIP_2 back to initial levels will depend upon PIP and other phosophoinositides within the cycle. The synthesis of PIP_2 is dependent

upon the phosphatidylinositol transfer proteins (PITP) family of proteins. PITPs function to transport phosphatidylinositol (PI) and phosphatidylcholine (PC) (Allen-Baume *et al.*, 2002) to cellular compartments where they can be further phosphorylated to PIP_2 by lipid kinases (Aikawa *et al.*, 1999). This couples PI to PIP_2 synthesis (Allen-Baume *et al.*, 2002). These proteins play important roles in receptor mediated PLC signalling (Kauffmann-Zeh *et al.*, 1995; Thomas *et al.*, 1993), by providing a continued source of plasma membrane PIP_2 by the transfer of PI from intracellular pools (Thomas *et al.*, 1993; Whatmore *et al.*, 1999). Wortmannin directly inhibits the re-synthesis of PIP_2 by inhibiting PI4K. The action at this site is a probably reason for the strong effect of wortmannin on slowing the rate of recovery of g_M after agonist-induced suppression. R59022 acts far 'upstream' from the site of action of wortmannin (Fig 1-1). If PITP are continuously replenishing PIP_2 levels in the membrane the action of R59022 would be presumed to be weak. It is not until substrate intermediates such as phosphatidic acid, CDP-DAG, PI and PIP are depleted that the effects of R59022 would be seen. The action of R59022 may reflect depletion of PIP_2 due to the depletion PI, which would explain why more agonist-induced 'turns of the cycle' were required to deplete the membrane concentrations of PIP_2 with R59022 than with wortmannin inhibition of PI4K (see Fig 1-1). This finding implicates the whole lipid and inositol cycle as being important for recovery of g_M after agonist-induced suppression. We have previously noted that the PLC inhibitor, U73122 produces a transient enhancement of g_M when it is applied to BFSG cells (Stemkowski *et al.*, 2002). Since this effect is not seen with the inactive isomer, U73343, it may reflect accumulation of PIP_2 in the membrane following inhibition of basal PLC activity.

Zhang et al (2003) reported that PIP_2 antibodies inhibit KCNQ2/3 channels in inside-out oocyte macropatches. This observation is consistent with the hypothesis that PIP_2 removal invokes channel closure. The observation that PIP_2 antibodies failed to affect steady-state g_M in BFSG neurons was therefore unexpected. Despite this, the antibody consistently attenuated the effect of both ATP and muscarine. One explanation for this may be that the antibodies protected PIP_2 from the action of PLC whilst preserving its interaction with M-channels. If this were so, the result would support the hypothesis that PIP_2 hydrolysis is required for agonist action. It does not, however, show

that PIP_2 removal invokes channel closure. Anti-PIP_2 antibodies were also used to examine the mechanism of transduction of g_M suppression by LHRH (see Chapter 3). In that case similar antibodies produced a different effect, namely inhibition of steady state g_M and LHRH-induced suppression of g_M, illustrating the variation between different commercially available antibodies. The findings from Chapter 3, do suggest that PIP_2 removal invokes channel closure. This issue is more fully addressed in Chapter 3.

Consistent with the 'PIP_2 depletion hypothesis' is the slowing of recovery of agonist responses by intracellularly-applied non hydrolyzable ATP analogs (Chen & Smith, 1992; Suh & Hille, 2002) (see Chapter 3). Also, the use of ATP-free internal pipette solutions with extracellular solutions which are free of metabolic substrates (glucose and pyruvate) promotes rundown of g_M, (Pfaffinger *et al.*, 1988; Simmons & Schneider, 1998; Tokimasa & Akasu, 1990 and see Chen & Smith, 1992; Simmons *et al.*, 1990). The dependence of ATP for both the maintenance of g_M (i.e, the prevention of 'run down') and the recovery from agonist-induced g_M suppression suggests that both effects are dependent upon a similar process. The impairment of ATP hydrolysis may reduce the activity of PI4K and PIPK, thus preventing the formation of PIP_2 which is required for g_M maintanance.

Modulation of g_M by PIP_2

The experiments of Zhang et al (2003), which studied effects of PIP_2 on putative M-channels in rat sympathetic neurons, were done using inside-out patches held at +10 mV. Similar experiments would be difficult in frog neurons as recordings at this voltage would be dominated by openings of maxi $g_{K,Ca}$ channels. The single channels recorded in SCG neurons by Zhang et al. (2003) were unlikely $g_{K,Ca}$ channels, as they exhibited conductances of 7 and 12 pS. While there exists the possibility that the channels examined were leak or delayed-rectifyer K^+ channels, the modulation by oxotremorine methiodide and PIP_2 makes this less likely. To avoid potential complications, whole-cell recordings instead of single M-channels were used in this thesis to study effects of PIP_2.

Although exogenous application of PIP_2 increases KCNQ2/3 currents in tsA 201 and COS-1 cells, it produced little effect on native g_M in BFSG neurones. This did not reflect insufficient delivery of PIP_2 from the patch pipette to the membrane as K_{ATP}

channels could be modulated by PIP_2 using our experimental arrangement. It is also unlikely that endogenous phosphatases degraded exogenously applied PIP_2 because the use of an internal fluoride-orthovanadate-pyrophosphate solution ('FVPP') (Huang *et al.*, 1998) did not increase the effectiveness of PIP_2. One explanation for the lack of effect of PIP_2 on native g_M may be that M-channels are already saturated with PIP_2 in the resting state, so that application of additional PIP_2 has no further effect. Yet the use of ATP free internal solutions and depleting endogenous PIP_2 by repeated applications of ATP in the presence of wortmannin, produced little or no g_M potentiation in BFSG neurones by the application of exogenous PIP_2. Other possibilities are that PIP_2 is especially tightly bound to the native M-channel or exogenously-applied PIP_2 has poor access to the PIP_2 binding site. These explanations are favoured as application of phosphatidylinositol 3,4,5 trisphosphate (PIP_3), which should be more effective than PIP_2 in modulation of channel activity (Fan & Makielski, 1997; MacGregor *et al.*, 2002), was without effect on g_M in BFSG cells. In the native neuron 'signalling rafts', or areas of high density of M-channels, receptors, accessory proteins and PIP_2 molecules may be present which do not occur when M-channels are expressed in artificial environments. In these signalling rafts, tight binding of PIP_2 to the native M-channel may also explain the inability of antibodies to disrupt the PIP_2-channel interaction (Zhang *et al.*, 1999). Despite this, the antibody consistently and very effectively attenuated the effect of both ATP and muscarine on g_M. This finding may be consistent with the 'PIP_2 depletion hypothesis' if the antibodies were protecting PIP_2 from the action of PLC whilst preserving its interaction with native M-channels. Because potentiation of KCNQ2/3 current was seen with PIP_2 in tsA201 and COS-1 cells and Al^{3+} clearly attenuated the current in patches from such cells, there may be differences between PIP_2 binding to expressed channels compared to native channels. One might speculate that the presence of a regulatory subunit, expressed only with native M-channels and not with KCNQ2/3 channels in expression systems, may stabilise PIP_2-channel binding. Additionally, it could be suggested that in C-cells there is no effect of PIP_2 on g_M, and thus there exists a difference in the regulation of M-channels between B- and C- cells. This possibility is rather unlikely due to the fact that multiple transmitters suppress g_M in both B- and C-cells, and one transmitter, LHRH, acts to suppress I_M in both cell types.

The affinity of M-channels towards PIP_2 may regulate the degree of suppression mediated by receptor stimulation. Mutation of KCNQ2 to reduce the sensitivity towards PIP_2 was recently found to reduce basal current of these mutants when expressed with wild-type KCNQ3 subunits. In addition, agonist application was found to cause greater suppression of the resulting current compared with wild-type currents suggesting that channels with reduced sensitivity towards PIP_2 are more susceptible to receptor-mediated inhibition (Zhang *et al.*, 2003).

Possible Role of DAG and Ca^{2+}

It is unclear whether PIP_2 depletion alone is sufficient to explain the robust suppression of g_M seen with all G_q-coupled agonists (Ikeda & Kammermeier, 2002). It is possible, for example that the release of DAG, as a result of agonist-activation of PLC may contribute to M-channel closure. DAG analogues suppress g_M in sympathetic neurones (Bosma & Hille, 1989; Chen *et al.*, 1994; Selyanko *et al.*, 1990). DAG may directly interact with M-channels (Chen *et al.*, 1994; Selyanko *et al.*, 1990), or may proceed via PKC (Bosma & Hille, 1989; Marrion, 1994). Recently, experiments in BFSG with the DAG analogue 1,2-dioctanyl-*sn*-glycerol (DOG) were not able to either support or disprove a direct link to M-channel modulation (Stemkowski *et al.*, 2002). PKC was previously believed to likely not transduce the agonist-induced signal to M-channel closure (Bosma & Hille, 1989; Marrion, 1994; Marrion, 1997; Selyanko *et al.*, 1990). However, its role has recently been re-examined, where it has been suggested that PKC does play a role in mediating muscarine-induced g_M suppression (hoshi *et al.*, 2003). Careful examination of the data present by Hoshi et al. (2003) however, suggests that PKC at best plays a small role in mediating the effects of muscarine on M-channels (see General Discussion). Within the 'PIP_2 depletion hypothesis', DAG may contribute to M-channel closure, in opposition to the maintaining effects of PIP_2. During agonist-induced g_M suppression, PLC-mediated hydrolysis would cause PIP_2 levels to fall in unison with an increase in DAG levels; both of these effects could cause M-channel closure. The present data make this hypothesis unlikely: (1) With the use of the DAG kinase inhibitor R59022, there would be an *immediate* and *long lasting* increase in the levels of DAG following agonist-suppression since the DAG produced would not be able

to be converted to phosphatidic acid. If DAG was the mediator of g_M suppression, there would be a more immediate M-channel suppression and a decrease in the rate of recovery initially in the presence of R59022 due to the decreased ability of the cell to remove DAG. However, this is not the case. Initially g_M levels are unchanged in the presence of R59022, and it is not until more agonist-induced turns of the cycle are executed that inhibition of DAG kinase affects g_M. This is possibly explained by the depletion of membrane concentrations of PIP_2 via the depletion of PIP_2 substrates (phosphatidic acid, CDP-DAG, PI and PIP) not by an increase in DAG levels. (2) If DAG was a mediator of M-channel closure, the application of PIP_2 would likely cause a decrease in resting g_M due to DAG production by the tonic hydrolysis of PIP_2 by PLC. This is not the case, as PIP_2 application to tsA-201 and COS-1 cells promotes maintenance of g_M. However, the presence of tonic PLC activity in BFSG may be questionable due to lack of effect of PIP_2 when PLC is inhibited by U73122.

The obligatory (Kirkwood *et al.*, 1991; Selyanko & Brown, 1996) or permissive role of Ca^{2+} (Beech *et al.*, 1991) in agonist-induced g_M suppression has frequently been discussed. Recently Ca^{2+} has been ruled out as the diffusable messenger mediating M1-muscarinic receptor modulation of KCNQ2/3 currents (Shapiro *et al.*, 2000). This confirms earlier reports that Ca^{2+} most likely serves a permissive role. However, if a Ca^{2+}-dependent isoform of PLC such as PLC-β (Haley *et al.*, 2000) is involved in the PIP_2 depletion mechanism, Ca^{2+} released via the conventional $InsP_3$ pathway may serve as a positive feedback mechanism during agonist-induced g_M suppression.

Conclusion

Experiments reported here and in the work of Suh and Hille (2002), and Zhang et al (2003) are consistent with the first viable hypothesis for the mechanism of agonist-induced g_M suppression to be proposed for some time. However, neither study provides unequivocal support for the 'PIP_2 depletion hypothesis' as questions can be raised as to the selectivity of the various inhibitors and the antibodies that have been used. The hitherto elusive transduction mechanism for agonist-induced g_M suppression may reflect a relatively commonplace mechanism for channel modulation as there have been recent reports indicating regulation of ion channels by PIP_2. A PLC-mediated PIP_2 depletion

mechanism has been reported to mediate muscarinic suppression of calcium-permeant TRPM7 channels in both cardiac cells and in an expression system (Runnels *et al.*, 2002). There is also recent evidence for regulation of Ca^{2+} channel function by PIP_2. Furthermore, the activation of membrane receptors that stimulate the hydrolysis of PIP_2 has been shown to cause Ca^{2+} channel inhibition in neurones and an oocyte expression system (Wu *et al.*, 2002). This and other data support the suggestion that the membrane phospholipid, PIP_2, favors an open state of the M-channel (Suh & Hille, 2002; Zhang *et al.*, 2003). Thus, when agonist-bound receptors interact with $G_{q/11}$ and PLC is activated, the concentration of PIP_2 in the vicinity of the M-channel decreases (Willars *et al.*, 1998), and channel closure is promoted. This process may be involved in the P2Y-induced g_M suppression and may explain the elusive transduction mechanism for the excitatory action of muscarine.

Table 2-1. Effect of PLC-inhibitor U73122 and its inactive isomer U73343 on muscarine responses.

	g_M suppression by 10 μM muscarine	g_M suppression by 10 μM muscarine in presence of inhibitor
U73122 (10 μM) (n=6)	80.8±4.2%	10.2±2.8% (P<0.0001)
U73343 (10 μM) (n=5)	81.7±5.4%	13.6±3.7% (P<0.0002) P=0.5 vs 10 μM U73122
U73122 (3 μM) (n=7)	70.3±3.9%	11.6±3.0% (P<0.0001)
U73343 (3 μM) (n=5)	84.0±2.6%	24.7±5.4% (P<0.005) P<0.05 vs 10 μM U73122

Paired t-tests were used to compare g_M suppression before and after application of inhibitors in each cell studied. Unpaired t-tests were use to compare differences between g_M suppression in groups of cells in the presence or absence of inhibitors. Whole-cell recordings were used in all cases.

Figure 2-1. Whole-cell recordings of effects of kinase inhibitors on ATP-induced M-current suppression. Chart recordings of steady-state I_M at –30 mV in BFSG B-neurons. Rapid downward deflections are responses to voltage ramps (from –30 to –110 mV) used to assess membrane conductance (Selyanko *et al.*, 1990). Records of voltage commands omitted for clarity. **(A).** Reversible reduction of I_M by 250 μM ATP and slowing of recovery of ATP response in presence of 10 μM wortmannin. Arrows indicate recovery phases of ATP responses. **(B).** Lack of effect of wortmannin on I_M in the absence of ATP application. **(C).** Lack of effect of the PI3K inhibitor, LY294002 (10 μM) on ATP responses. **(D).** Effect of the myosin light chain kinase inhibitor ML-7 (10 μM). This substance produces a rapid attenuation of steady state I_M (gray arrow). Although the amplitude of the ATP response is attenuated, the rate of recovery is unchanged.

Figure 2-1

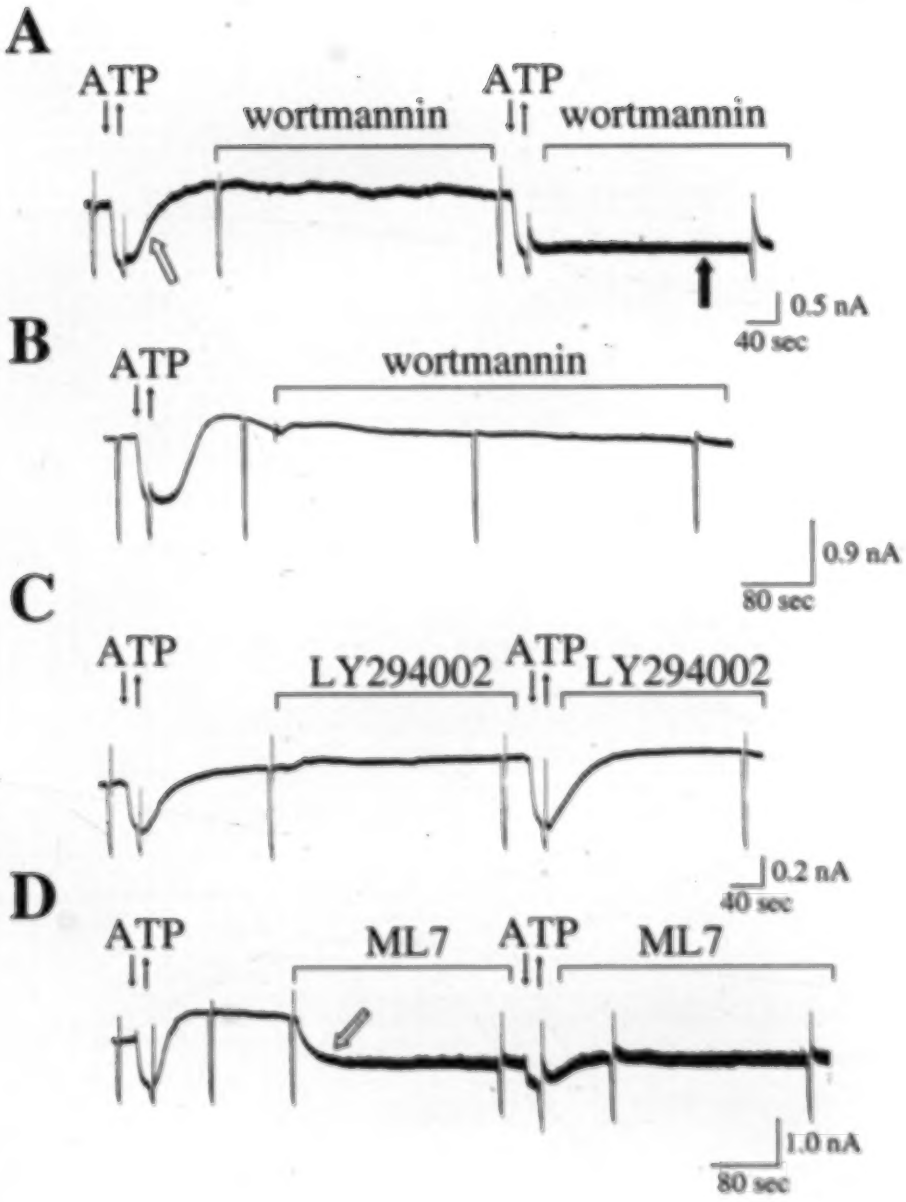

A
ATP
wortmannin
ATP
wortmannin
0.5 nA
40 sec
B
ATP
wortmannin
0.9 nA
80 sec
C
ATP
LY294002
ATP
LY294002
0.2 nA
40 sec
D
ATP
ML7
ATP
ML7
1.0 nA
80 sec

Figure 2-2. Current-voltage plots derived from 4.5 sec ramp commands to measure membrane conductance, as explained in the methods section. Suppression of current positive to -75 mV (i.e., in the M-conductance (g_M) range) by ATP (250 μM).

Figure 2-2

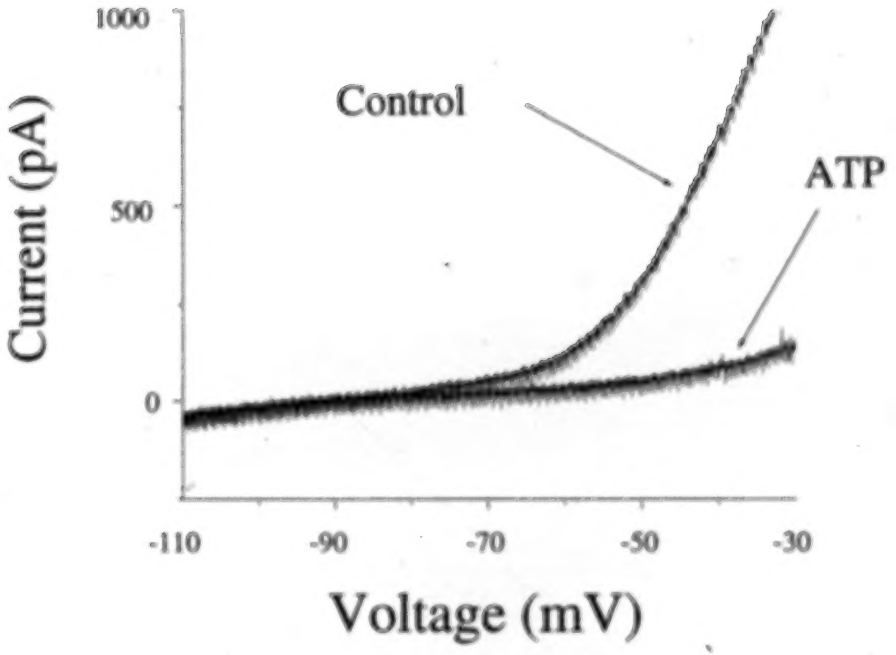


Figure 2-3. Bar graph to show that wortmannin (10 μM) slows the time course of recovery of the ATP response but LY 294002 (10 μM), ML-7 (10 μM) and DMSO control does not.

Figure 2-3

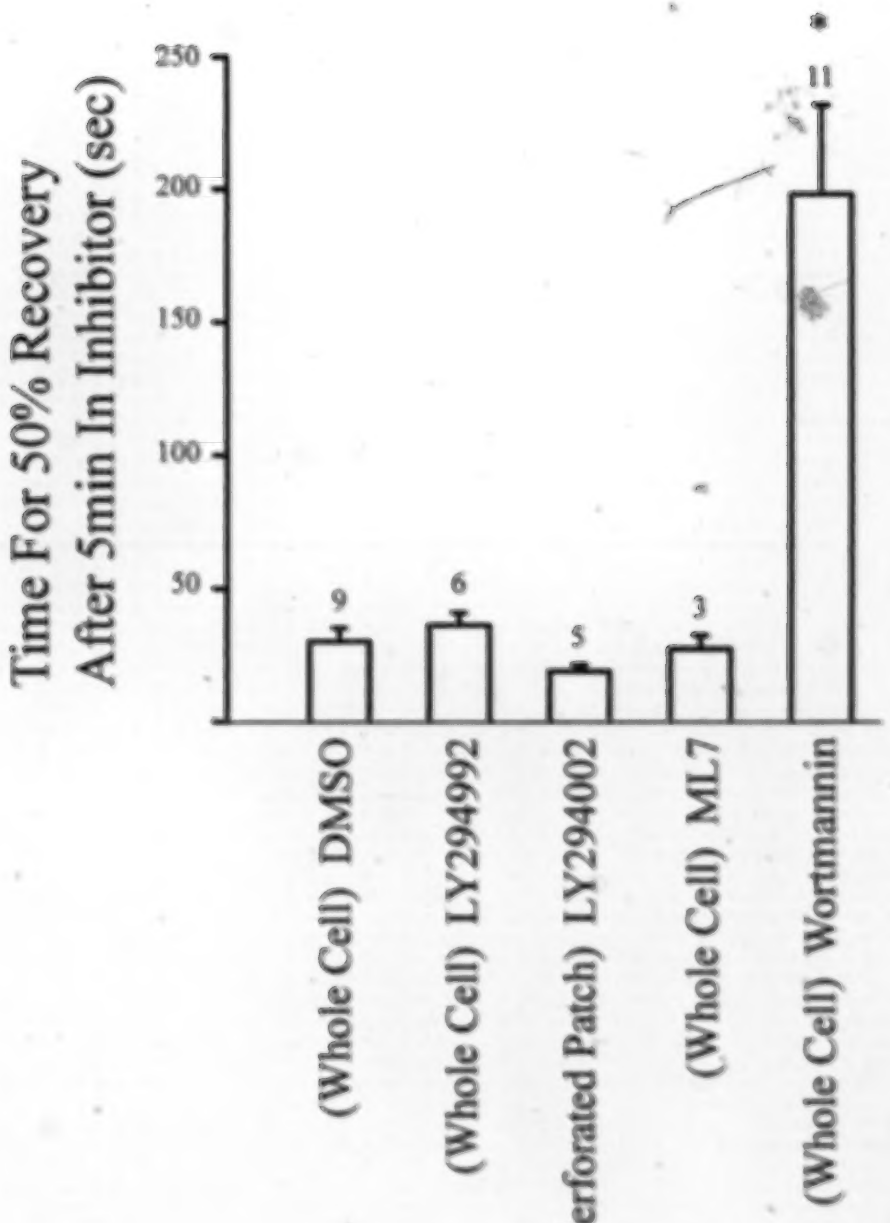

Time For 50% Recovery
After 5min In Inhibitor (sec)
250
200
150
100
50
9
6
5
3
11
*
(Whole Cell) DMSO
(Whole Cell) LY294992
(Perforated Patch) LY294002
(Whole Cell) ML7
(Whole Cell) Wortmannin

Figure 2-4. Whole-cell Recordings of Effects of the Diacylglycerol Kinase Inhibitor, R59022 on ATP-induced M-current Suppression. **(A)** Chart recordings of steady-state I_M at –30 mV in BFSG B-neurons. Rapid downward deflections are responses to voltage ramps (from –30 to –110mV) used to assess membrane conductance. Records of voltage commands omitted for clarity. Response to 250 μM ATP applied before R59022 recovers rapidly but rate of recovery progressively decreases during superfusion of 10 μM R59022. Arrows indicate recovery phases of ATP responses. **(B)** Summary of data from all cells tested. Times for 50 % recovery of ATP responses recorded before and after 5 and 10 min in R59022. Note progressive slowing of recovery of ATP responses recorded in the presence of R59022 compared to controls recorded without drug. Error bars show SEM, n's range from 6-9 measurements for each point.

Figure 2-4

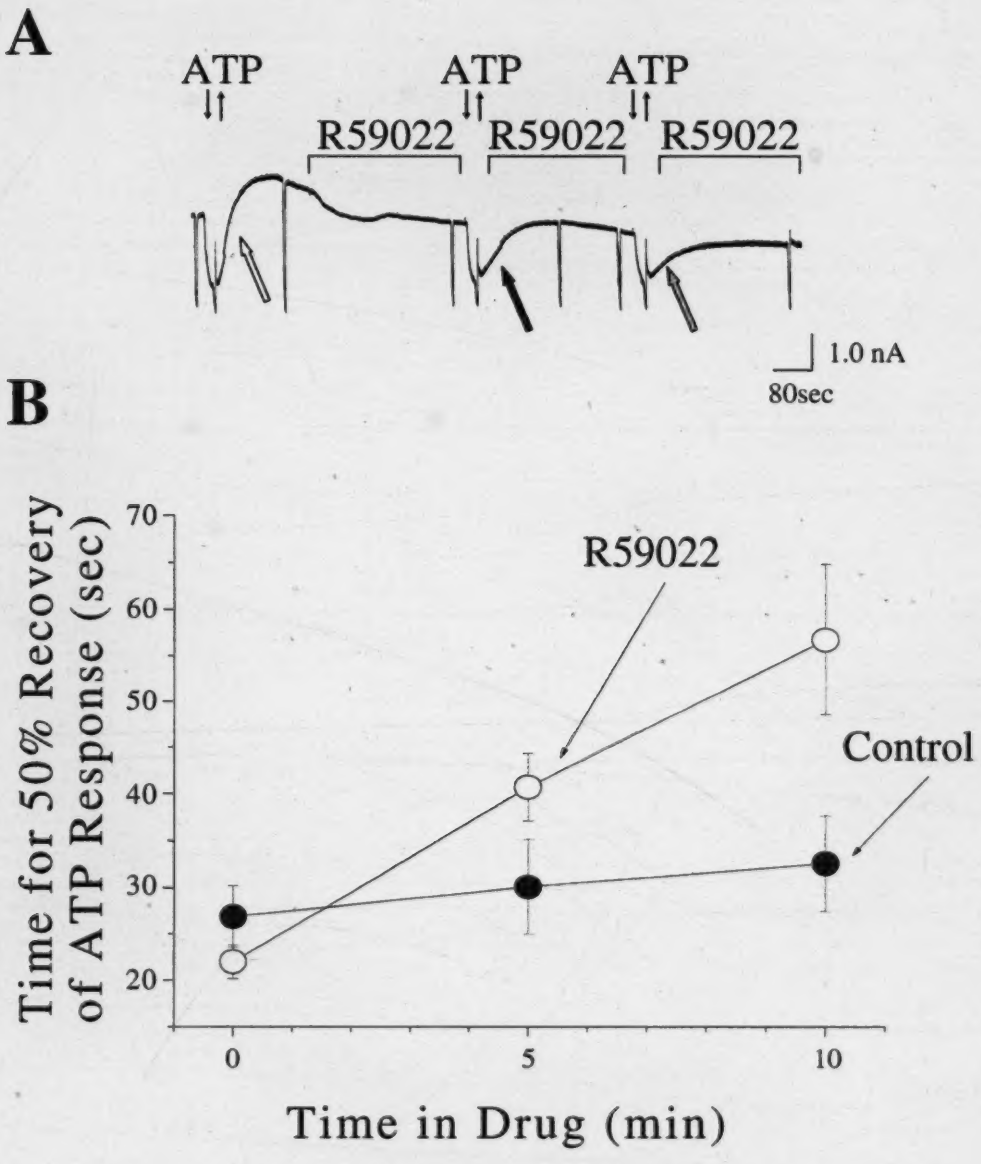
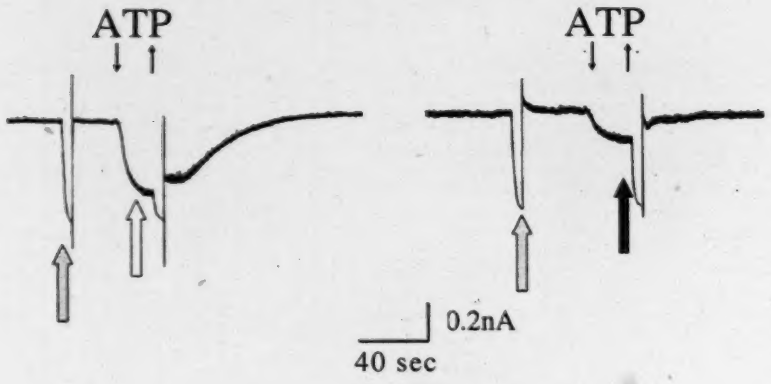

A
ATP
ATP
ATP
R59022
R59022
R59022
1.0 nA
80sec
B
Time for 50% Recovery of ATP Response (sec)
70
60
50
40
30
20
R59022
Control
0
5
10
Time in Drug (min)

Figure 2-5. Whole-cell Recordings to Illustrate Effect of PIP_2 Antibody on ATP-Induced M-current Suppression. Chart recordings of steady-state I_M at –30 mV in BFSG B-neurons. Rapid downward deflections are responses to voltage ramps (from –30 to –110 mV) used to assess membrane conductance. Records of voltage commands omitted for clarity. **(A)** Responses to 250 μM ATP recorded with a pipette containing 1:100 PIP_2 antibody. *Left hand panel;* response recorded 5 min after establishing whole-cell recording conditions. *Right hand panel;* response recorded after 25 min of recording. Note preservation of steady-state I_M demonstrated by unchanged amplitude of current responses to voltage ramps (gray arrows) but decreased effectiveness of ATP demonstrated by smaller amplitude response (black arrows compared to white arrows). **(B)** Effect of recording using a pipette containing horse serum (1:00 in internal solution) as control experiment for data presented in **(A)**.

Figure 2-5

A

1:100 anti-PIP_2 antibody



B

1:100 horse serum

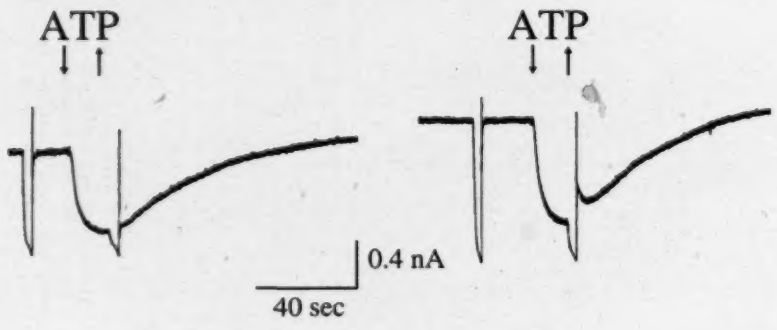


Figure 2-6. Anti-PIP_2-antibodies protect I_M from ATP-induced Suppression. **(A)** Bar graph to show the lack of effect of PIP_2-antibodies on basal, steady state I_M. **(B)** Dialysis of cells for 25 min with anti-PIP_2-antibodies protected I_M from ATP-induced suppression, an effect which was not seen with horse serum controls.

Figure 2-6

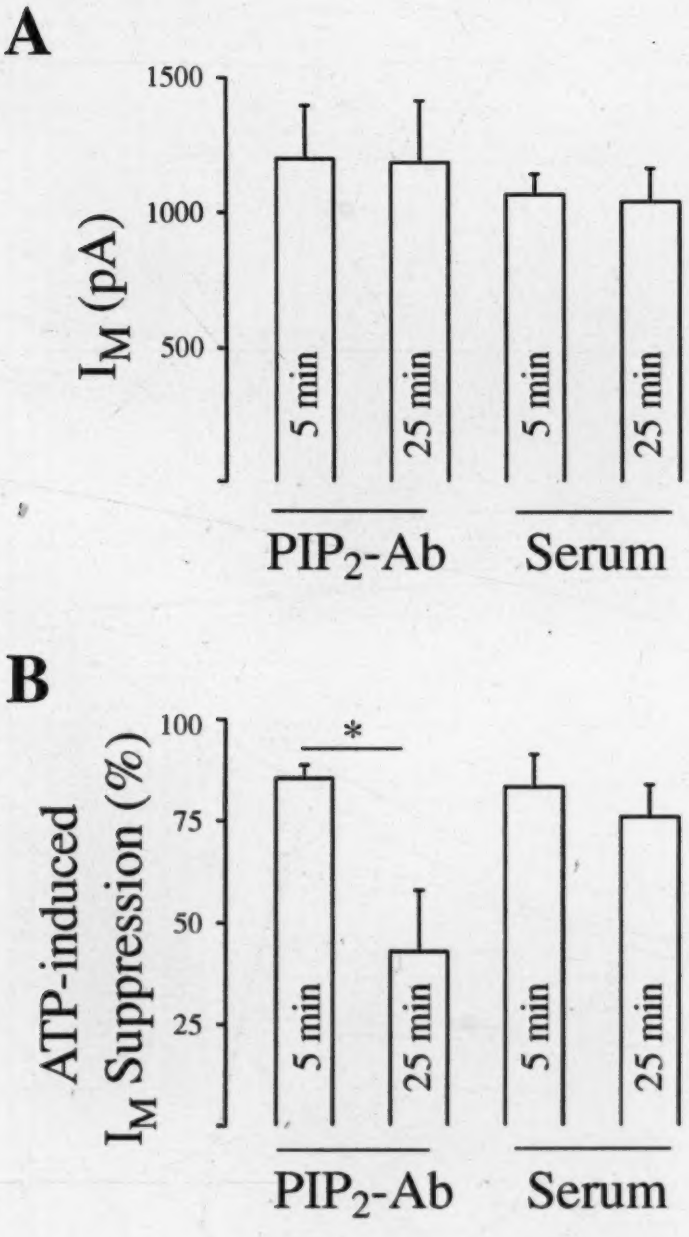

A
1500
1000
500
I_M (pA)
5 min
25 min
5 min
25 min
PIP_2-Ab
Serum
B
100
75
50
25
ATP-induced
I_M Suppression (%)
*
5 min
25 min
5 min
25 min
PIP_2-Ab
Serum

Figure 2-7. Modulation of cytoplasmic ATP sensitivity of K_{ATP} (Kir 6.2/SUR1) channels by 20 μM PIP_2. Currents recorded at +80 mV from inside-out patches at 30sec and 5min after pulling patches from tsA-201 cells (*see inset* for diagram of recording arrangement). Note lack of altered ATP sensitivity of currents recorded with a control pipette after 5 min **(A)** as opposed to the reduced sensitivity to the inhibitory effects of ATP of currents **(B)** recorded with a pipette containing 20 μM PIP_2 (*see inset* for diagram of recording arrangement).

Figure 2-7

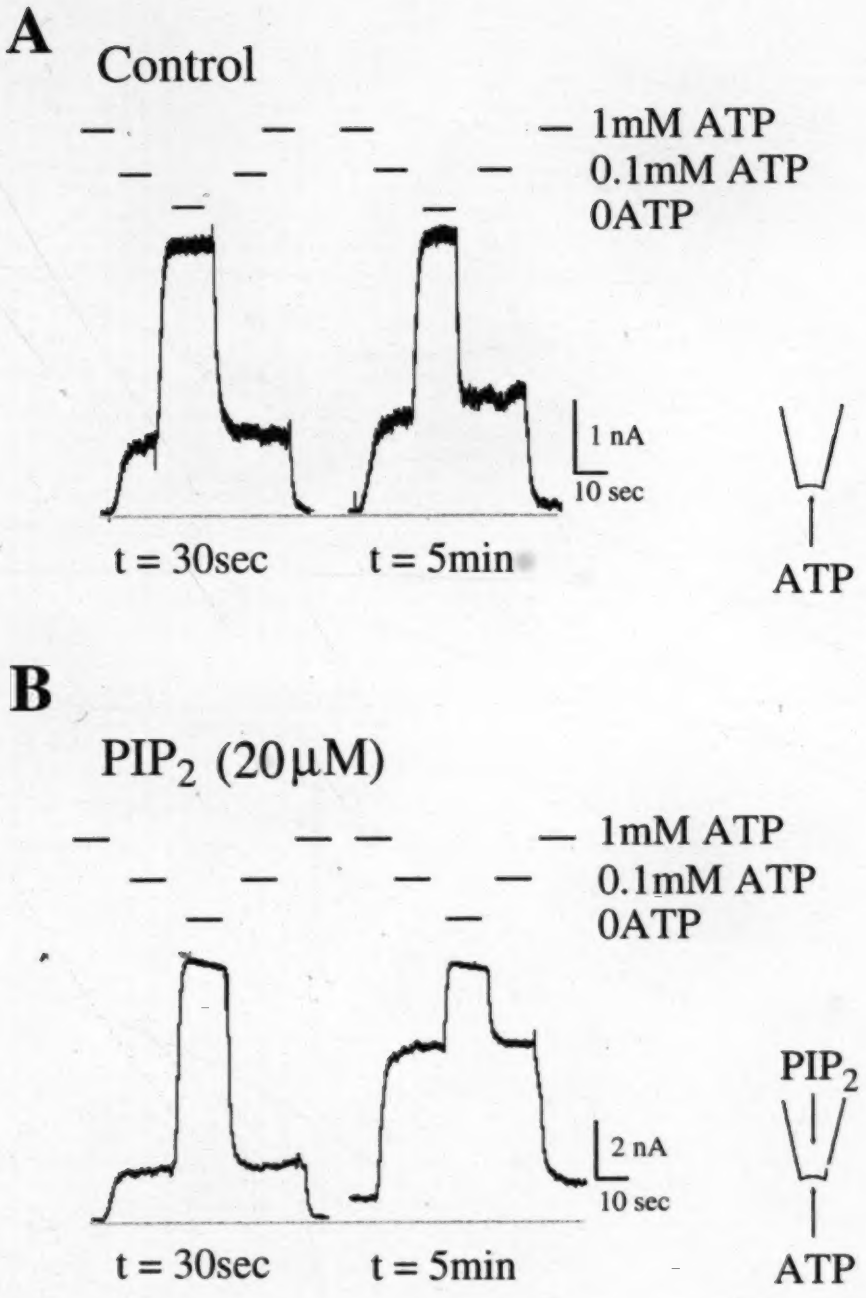

A
Control
1mM ATP
0.1mM ATP
0ATP
1 nA
10 sec
t = 30sec
t = 5min
ATP
B
PIP2 (20μM)
1mM ATP
0.1mM ATP
0ATP
2 nA
10 sec
t = 30sec
t = 5min
PIP2
ATP

Figure 2-8. Modulation of expressed KCNQ2/3 current by PIP_2 (20μM). Whole-cell recordings of expressed KCNQ2/3 current from tsA-201 and COS-1 cells. **(A)** Comparison of the time course of run down of KCNQ2/3 currents in control tsA-201or COS cells (n=21) compared with cells studied with a pipettes containing 20 μM PIP_2 (n=10) ($P<0.05$ for points labelled *). *Inset;* Relaxation and reactivation of KCNQ2/3 channel currents in response to a voltage step from –20 to –50 mV. Currents recorded 30s and 5min after initiating whole cell recording. **(B)** and **(C)** Responses to ramp commands from –80 to +50 mV to show current-voltage characteristics of KCNQ2/3 channels over a broad voltage range. Currents recorded 30 s and 5 min after initiating whole-cell recording. Note rundown of current in cell illustrated in (B) and persistence of current in cell illustrated in (C) that was studied with a pipette that contained 20 μM PIP_2 (*see inset* for diagram of recording arrangement).

Figure 2-8

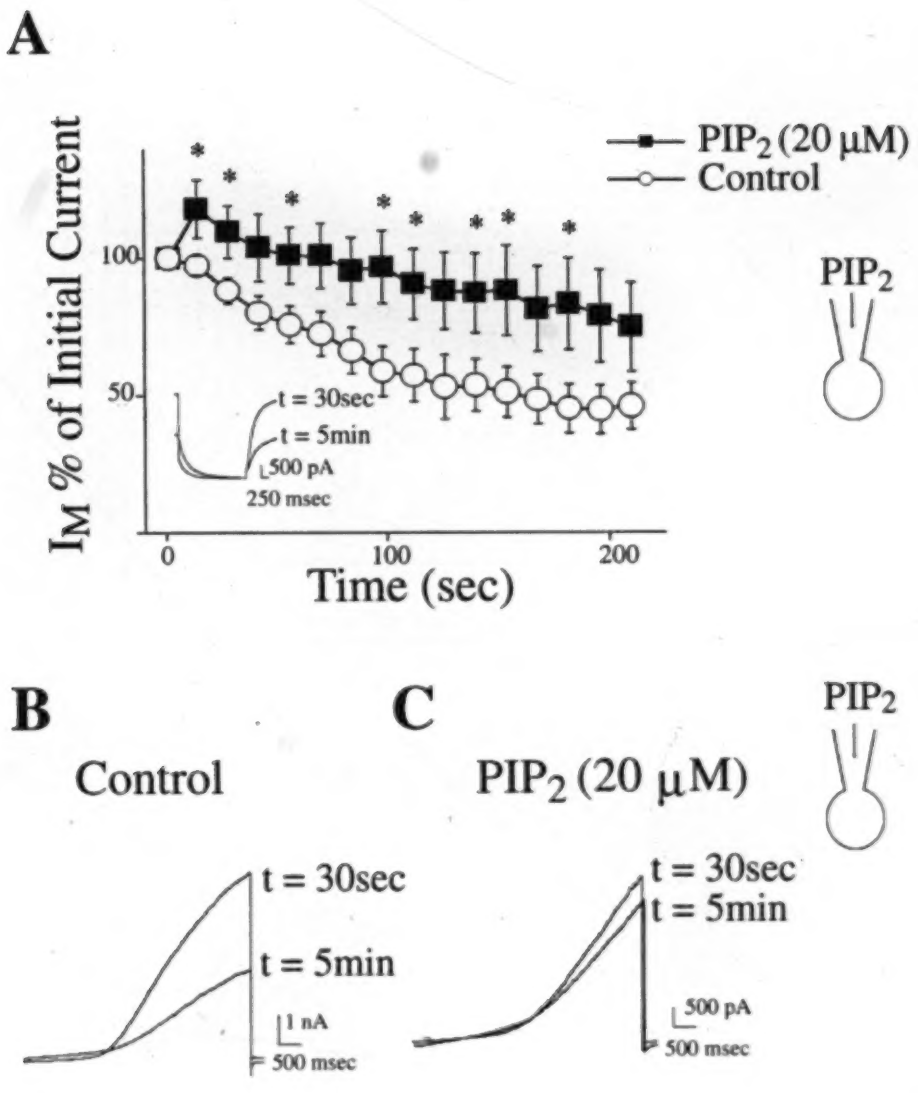

A
PIP2 (20 μM)
Control
PIP2
IM % of Initial Current
100
50
t = 30sec
t = 5min
500 pA
250 msec
0
100
200
Time (sec)
B
Control
t = 30sec
t = 5min
1 nA
500 msec
C
PIP2 (20 μM)
PIP2
t = 30sec
t = 5min
500 pA
500 msec

Figure 2-9. Effect of PIP_2 on J_M in BFSG C-neurons. **(A)** Whole-cell recordings from BFSG C-neurons. Comparison of the first 5min of recordings of I_M in control BFSC C-neurons (ATP-free intracellular solution n=9) compared with cells studied with a pipettes containing 100μM PIP_2 (n=11) ($P<0.05$ for points labelled *). **(B)** and **(C)** Change in I_M over 250 seconds of C-neurons dialyzed with control internal, or internal containing 100 μM PIP_2.

Figure 2-9

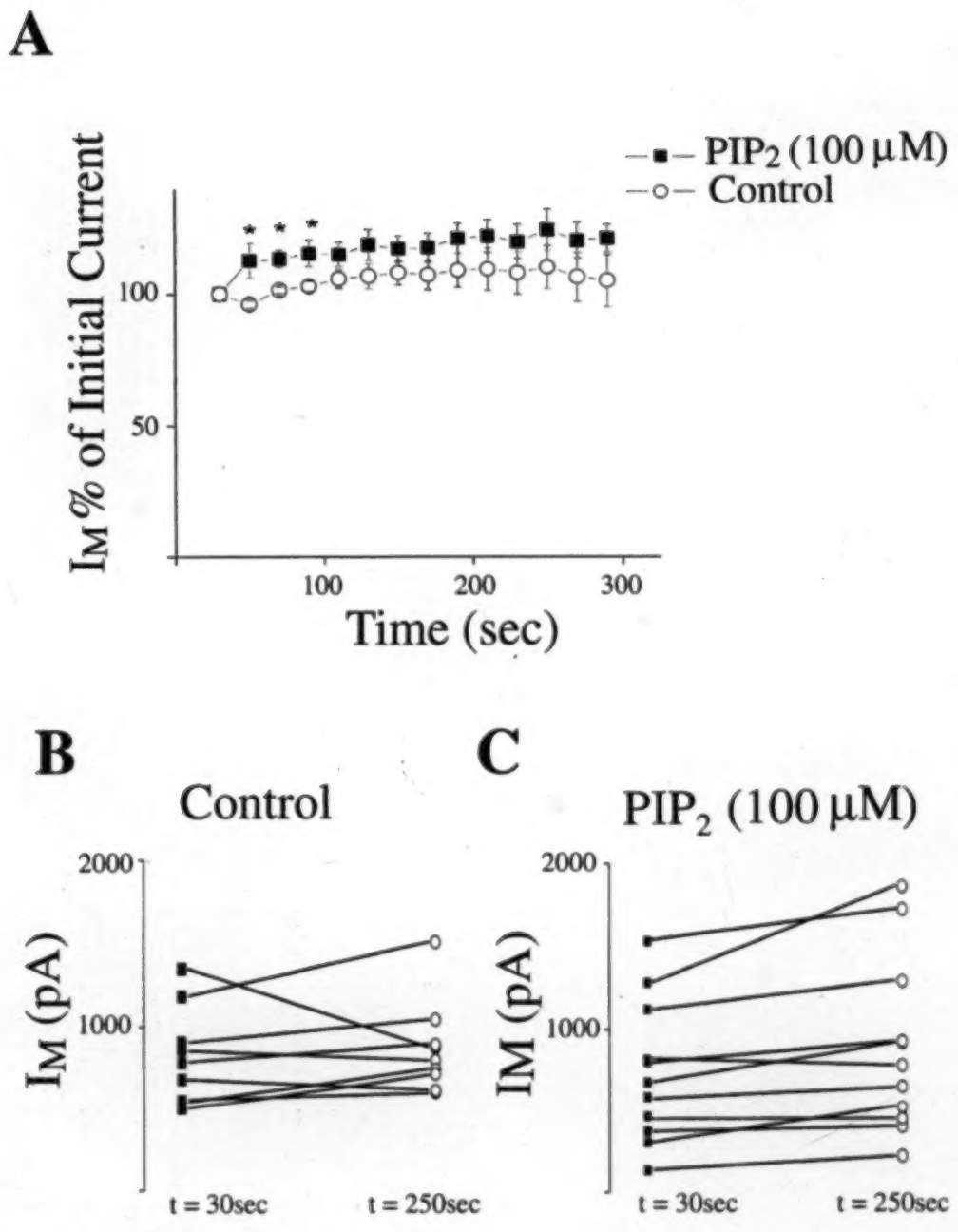

A
PIP2 (100 μM)
Control
IM % of Initial Current
100
50
100
200
300
Time (sec)
B
Control
C
PIP2 (100 μM)
IM (pA)
2000
1000
t = 30sec
t = 250sec
IM (pA)
2000
1000
t = 30sec
t = 250sec

Figure 2-10. Investigations into the limited effect of PIP_2 on I_M in BFSG C-neurons. Bar graphs showing the % 'run-up' (i.e., % of initial current) of I_M after 4.5 of recording compared to initial I_M levels. Both PIP_2 and PIP_3 were used at a concentration of 20 μM. Paired t-tests were used to compare current amplitudes at 30s and 4.5 min in each cell studied. Unpaired t-tests were use to compare differences between groups of cells after 4.5 min of recording under various experimental conditions. Whole-cell recordings were used in all cases. 2 mM ATP was included in the intracellular solution in all cases except ATP-Free, and solutions containing FVPP.

Figure 2-10

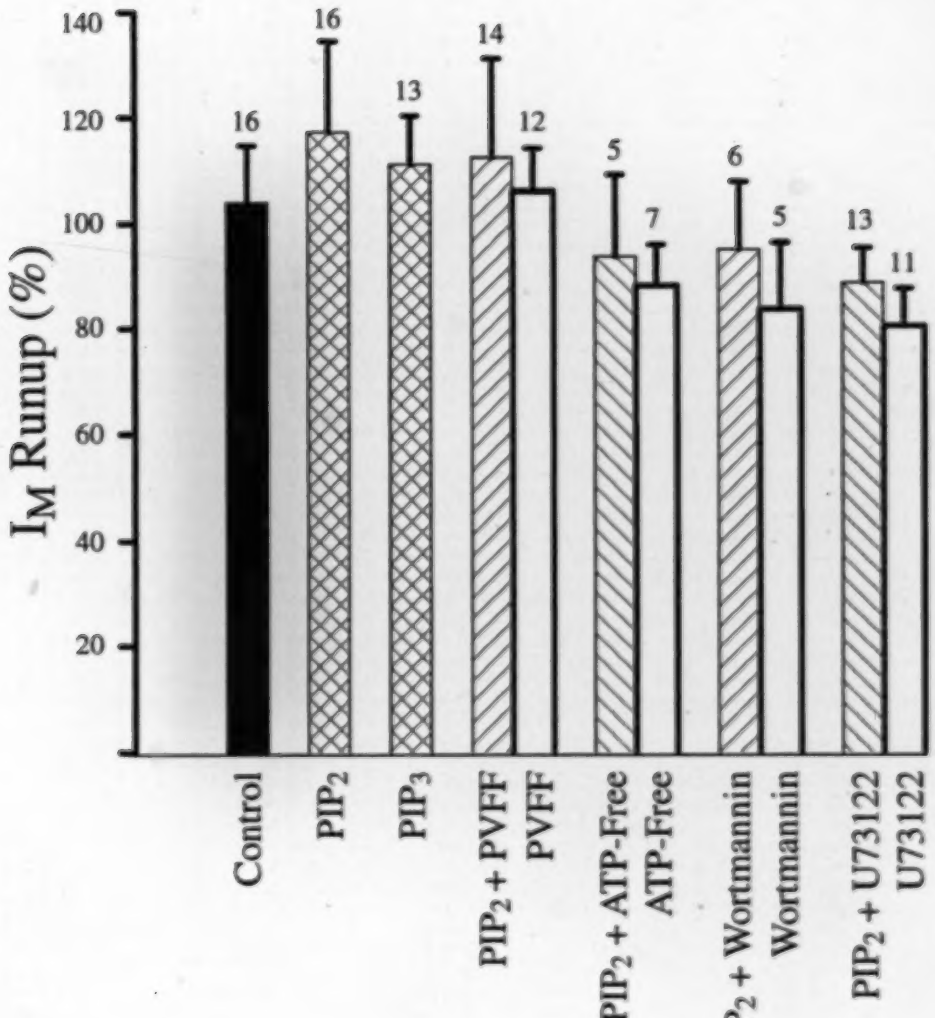

I_M Runup (%)
140
120
100
80
60
40
20
16
16
13
14
12
5
7
6
5
13
11
Control
PIP2
PIP3
PIP2 + PVFF
PVFF
PIP2 + ATP-Free
ATP-Free
PIP2 + Wortmannin
Wortmannin
PIP2 + U73122
U73122

Figure 2-11. Effects of Al^{3+} and Intracellularly Applied ATP on KCNQ2/3 Current in Excised Patches. Recording of KCNQ2/3 current in inside-out patch excised from a tsA-201 cell. Pipette solution contained 2.5 mM K^+ and solution outside the patch contained 110 mM K^+; E_K = -95 mV. Holding potential was 0 mV so current through KCNQ2/3 channels is towards pipette (*i.e.* inward according to usual convention). Bath application of 50 μM $AlCl_3$ (to the cytoplasmic face of the membrane) reduces current by about 100 pA and current is partly restored by application of 1 mM ATP (also to the cytoplasmic side of the membrane, see inset for diagram of recording arrangement). Bars show +80 mV step changes in holding potential across the patch from 0 mV to increase driving force and to measure conductance. Note decrease in conductance in presence of Al^{3+} and restoration of conductance with ATP.

Figure 2-11

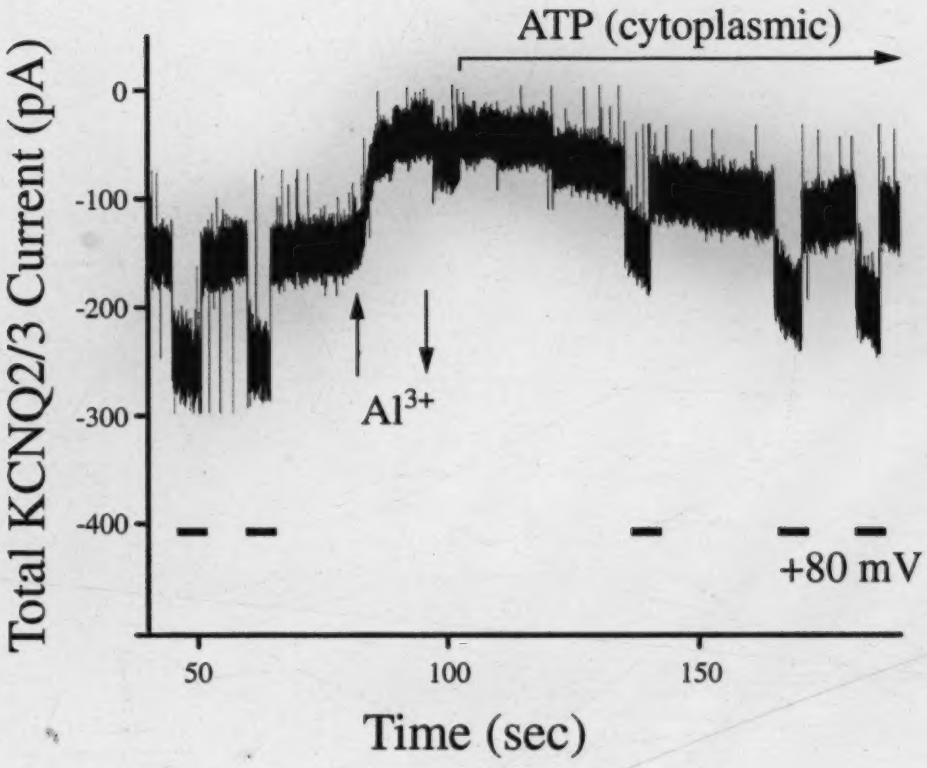

ATP (cytoplasmic)
Total KCNQ2/3 Current (pA)
0
-100
-200
-300
-400
Al^{3+}
+80 mV
50
100
150
Time (sec)

Figure 2-12. Whole-cell Recordings from BFSG Neurons to Illustrate Effects of PLC Inhibition on Muscarine-Induced g_M Suppression. Chart recordings of steady-state I_M at –30 mV in BFSG B-neurons. Rapid downward deflections are responses to voltage ramps (from –30 to –110 mV) used to assess membrane conductance. Records of voltage commands omitted for clarity. **(A)** Reversible reduction of I_M by 10 μM muscarine and inhibition of this effect by the PLC inhibitor, U73122 (10 μM). **(B)** Reversible reduction of I_M by 10 μM muscarine and inhibition of this effect by the inactive isomer, U73343 (10 μM).

Figure 2-12

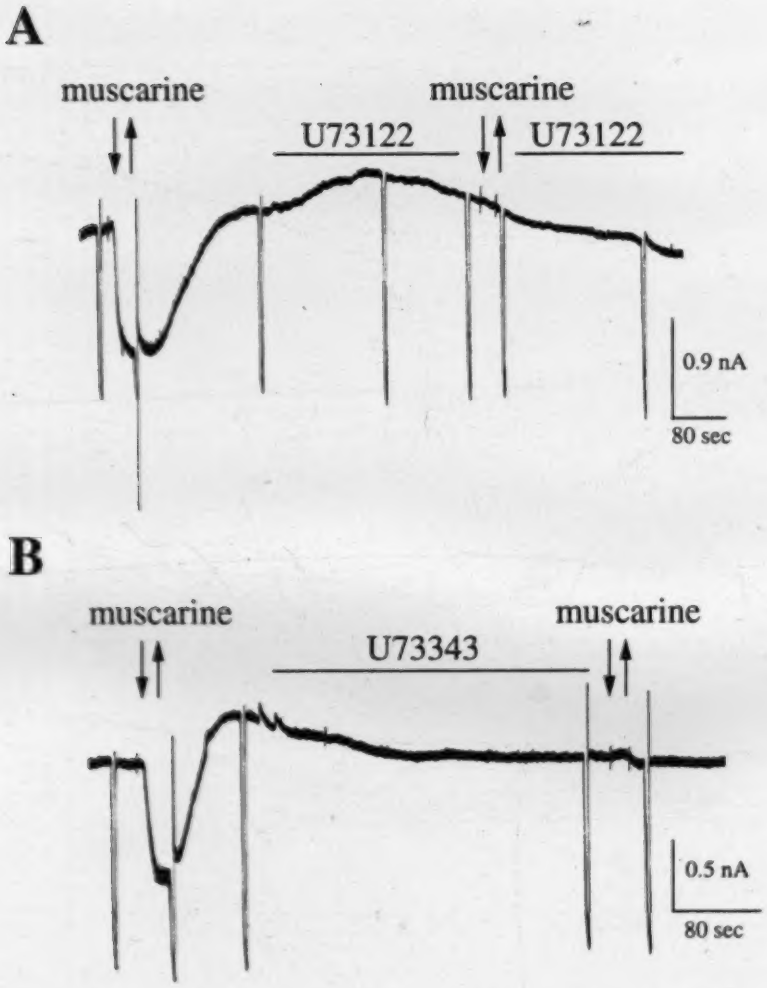

A
muscarine
muscarine
U73122
U73122
0.9 nA
80 sec
B
muscarine
muscarine
U73343
0.5 nA
80 sec

Figure 2-13. Effects of different concentrations of U73343 or U73122 on g_M suppression by 10 μM muscarine.

Figure 2-13

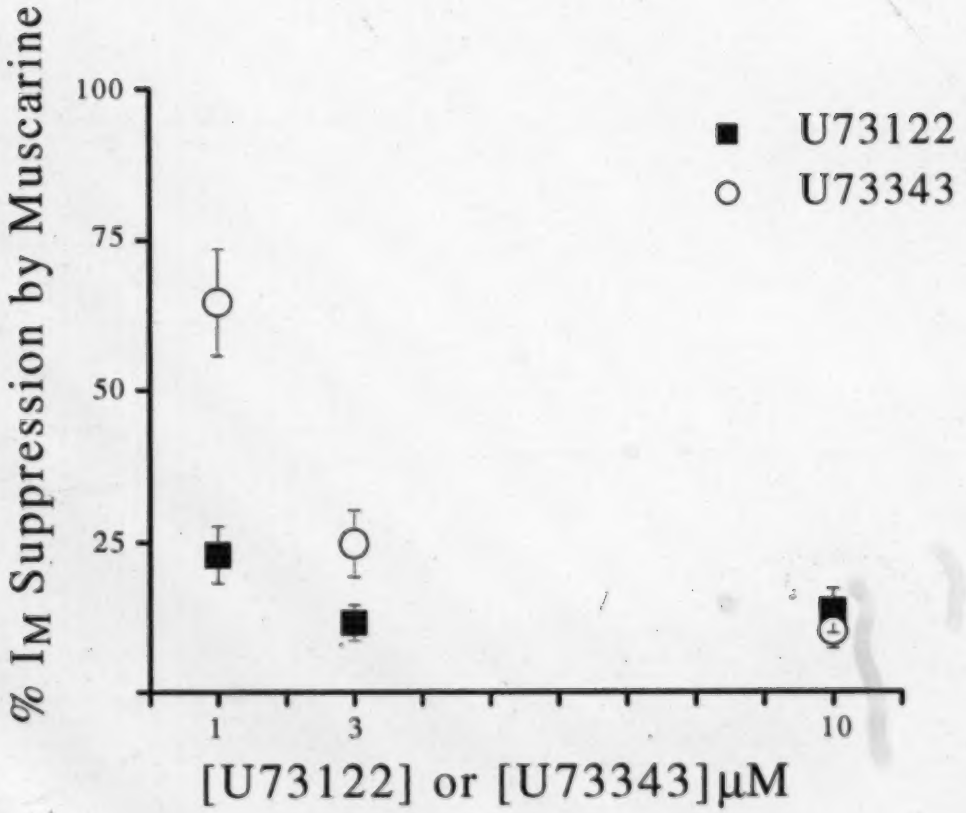

% IM Suppression by Muscarine
100
75
50
25
1
3
10
[U73122] or [U73343]μM
U73122
U73343

Figure 2-14. Whole-cell Recordings from BFSG Neurons to Illustrate Effects of Wortmannin on Muscarine-Induced g_M Suppression. Chart recordings of steady-state I_M at –30 mV in BFSG B-neurons. Rapid downward deflections are responses to voltage ramps (from –30 to –110 mV) used to assess membrane conductance. Records of voltage commands omitted for clarity. Slowing of recovery of response to 10 μM muscarine by 10 μM wortmannin. Arrows indicate recovery phases of muscarine responses.

Figure 2-14

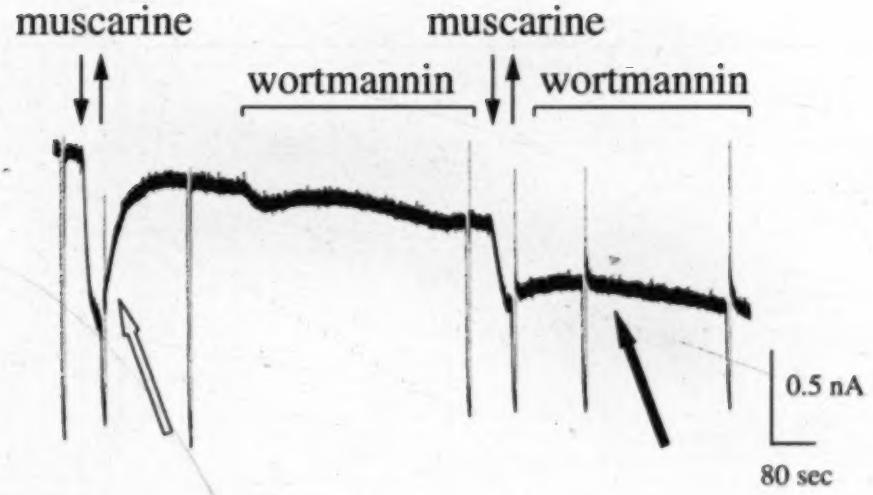
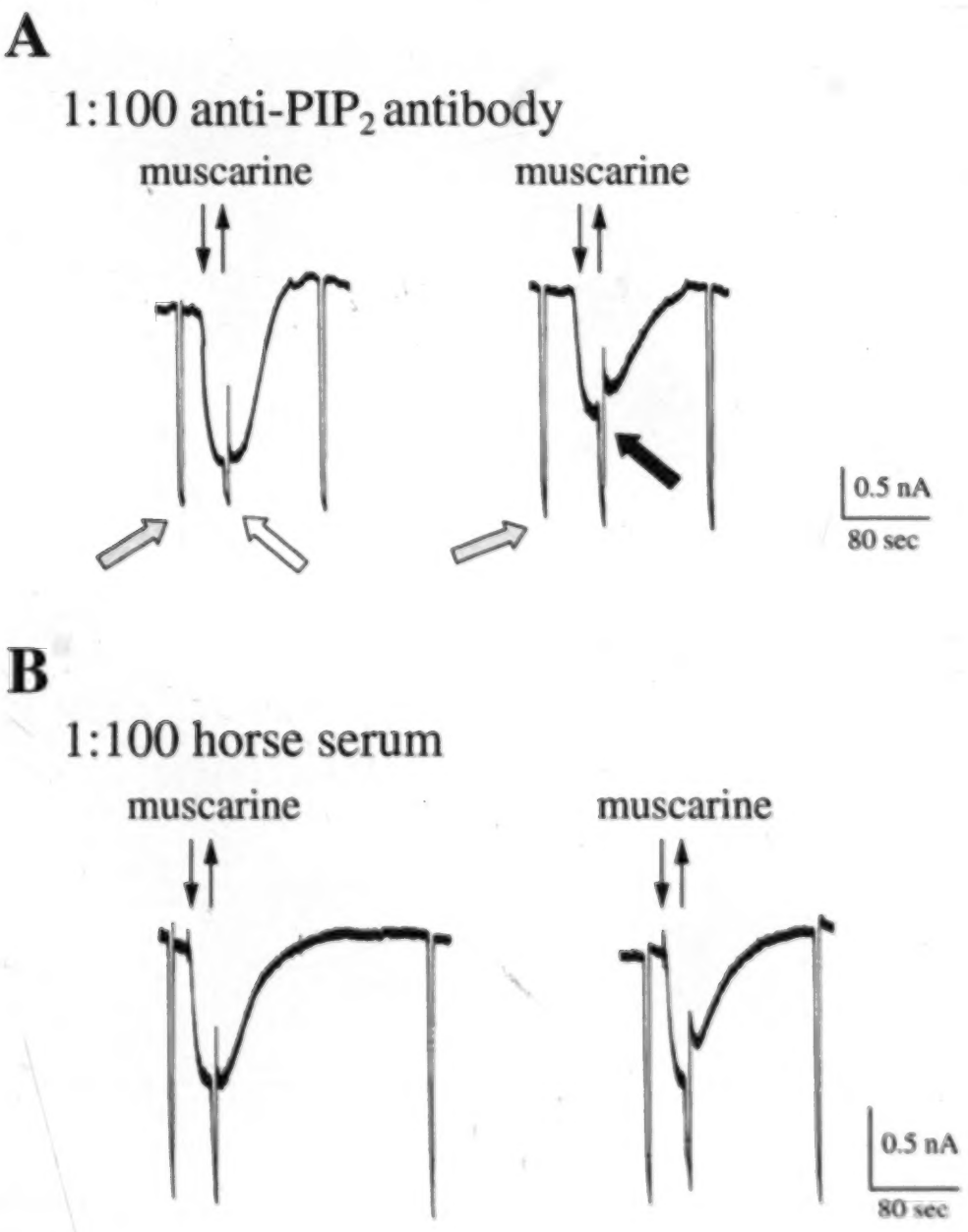


Figure 2-15. Whole-cell Recordings from BFSG Neurons to Illustrate Effects of PIP_2 Antibodies on Muscarine-Induced g_M Suppression. Chart recordings of steady-state I_M at –30 mV in BFSG B-neurons. Rapid downward deflections are responses to voltage ramps (from –30 to –110 mV) used to assess membrane conductance. Records of voltage commands omitted for clarity. **(A)** Responses to 2 μM muscarine recorded with a pipette containing 1:100 PIP_2 antibody. *Left hand panel;* response obtained after 5min of recording. *Right hand panel;* response recorded after 25 min of recording. Note preservation of steady-state g_M demonstrated by unchanged amplitude of current response to voltage ramps (grey arrows) but decreased effectiveness of muscarine demonstrated by smaller amplitude response (black arrows compared to white arrows). **(B)**. Control experiment for **(A)** done with pipette containing 1:100 horse serum

Figure 2-15


A
1:100 anti-PIP2 antibody
muscarine
muscarine
0.5 nA
80 sec
B
1:100 horse serum
muscarine
muscarine
0.5 nA
80 sec

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Chapter 3

Luteinizing Hormone Releasing Hormone-Mediated M-Channel Closure Involves Depletion of Phosphatidylinositol 4,5 Bisphosphate

A version of this chapter is in revision
for the Journal of Neurophysiology

INTRODUCTION

As discussed in the General Introduction, the M conductance (g_M) is a voltage- and time- dependent, non-inactivating K^+ current The results from Chapter 2, are consistent with the hypothesis that ATP and muscarine induced M-channel closure in BFSG neurons involves the PLC-mediated depletion of PIP_2. These findings support recent work indicating that the suppression of M-channels in SCG neurons and KCNQ2/3 channels in expression systems by muscarine also involves this same mechanism (Suh & Hille, 2002; Zhang *et al.*, 2003). However not all agonists which suppress g_M do so by the same mechanism. The suppression of g_M by bradykinin, and nucleotides in SCG neurons, occurs via Ca^{2+} release from IP_3-sensitve Ca^{2+} stores (Cruzblanca *et al.*, 1998; del Rio *et al.*, 1999; Bofill-Cardona *et al.*, 2000; Shapiro *et al.*, 2000; Stemkowski *et al.*, 2002; Suh & Hille, 2002).

A major area of interest of this thesis is determining both the acute and long-term actions of a single neurotransmitter (LHRH) in the same defined system. LHRH acts acutely to mediate suppression of g_M (Brown & Adams, 1980; Jones, 1987). However, the mechanisms responsible remain unknown. This chapter examines the mechanism of LHRH-induced g_M suppression, to see if LHRH mediates M-channel closure like bradykinin, by a mechanism involving Ca^{2+} release from IP_3 sensitive stores, or like muscarine, by a mechanism involving the PLC-mediated depletion of PIP_2.

METHODS

Methods used in this chapter were the same as described for experiments involving BFSG neurons in 'Methods' of Chapter 2.

Drugs and Chemicals

In all cases LHRH was used at a 100 nM concentration. Drugs were applied to BFSG neurons using a rapid superfusion system that was constructed from 0.8 mm diameter polyimide tubes. Aliquots of U73122 and U73343 were first dissolved in chloroform which was then allowed to evaporate under a stream of nitrogen to yield a filmy residue which was stored at –20°C until the day of the experiment when it was dissolved in fresh DMSO. LY294002, wortmannin and chelerythrine-Cl_2 were also

dissolved in fresh DMSO. Heparin was dissolved in internal solution. Serial dilutions of drugs in external solution were arranged so that the final concentration of DMSO in solutions applied to cells was <0.1%. Acute application of 0.1 % DMSO on B-neurons had no noticeable effect on their electrophysiological properties (see Chapter 2).

LHRH (Chicken II-GnRH) and bradykinin were obtained from Bachem (Torrance, CA, USA). U73122, U73343 and wortmannin were from Biomol (Plymouth Meeting, PA, USA), PIP_2 Antiserum was purchased from Assay Designs Inc. (Anne Arbor, MI, USA). All other chemicals were from Sigma (Oakville, ON, Canada). Data are expressed as mean ± S.E.M. and significance of difference assessed using Student's two tailed *t*-test or ANOVA (Student-Newman-Keuls post-hoc comparison). Paired tests were used for pairs of observations on the same cell and unpaired tests were used for comparison of data from groups of cells. Data were considered significantly different when $p<0.05$.

RESULTS

LHRH Suppresses g_M via the activation of PLC

Like many other receptors that mediate suppression of g_M, the LHRH receptor most likely couples to $G_{q/11}$ to cause the activation of phospholipase C (PLC) (Pfaffinger *et al.*, 1988; Marrion, 1997). To test the role of PLC in mediating g_M suppression by LHRH, BFSG B-neurons were challenged with LHRH (100 nM) in the presence of the PLC inhibitor U733122 (10 μM) or the inactive control compound U73343 (10 μM). A typical experiment is illustrated in Fig 3-1 A and B which shows chart recordings of steady state I_M at -30 mV interrupted by responses to voltage ramps to -110 mV which were used to monitor agonist-induced changes in membrane conductance. These ramps produce I-V plots such as those illustrated in Fig 3-2. Control application of LHRH produced 75.3±4.5 % (n=5) suppression of g_M which was significantly reduced to 14.6±4.2 % (n=5) ($p<0.001$) after a 4.5 min application of 10 μM U733122 (Fig 3-2A and 3-3). This effect was not mimicked by a similar treatment with the inactive analogue U73343 (10 μM), as control application of LHRH produced 82.7±2.4 % (n=6) suppression which did not differ from the 77.5±4.4 % (n=6) suppression produced by LHRH in the presence of 10 μM U73343 ($p>0.05$) (Fig 3-2B and 3-3). These results

suggest that LHRH-mediated suppression of g_M involves the activation of PLC. As previously reported, 10 μM U733122 also caused a reproducible potentiation of g_M which was not seen with the inactive U73343 (Stemkowski *et al.*, 2002; Suh & Hille, 2002), which may indicate the presence of a basal activity of PLC that functions to suppress g_M in the absence of receptor activation.

LHRH suppression of g_M does not involve PKC, IP_3 nor Ca^{2+}

Activation of PLC mediates the hydrolysis of PIP_2 into DAG and IP_3. These 'downstream' products can lead to increases in intracellular Ca^{2+} via IP_3 sensitive stores and the activation of protein kinase C (PKC). We tested the possible involvement of these pathways in mediating LHRH-induced suppression of g_M.

A previously established protocol, involving dialysis of BFSG B-neurons with 10 μM chelerythrine plus treatment with 5 μM extracellular chelerythrine, was used to inhibit PKC (Stemkowski *et al.*, 2002). The initial suppression of g_M by LHRH, recorded within 1min of attaining whole-cell recording conditions was 71.5±4.6 % (n=6). This was unchanged after 8min of recording with intracellular 10 μM chelerythrine plus 7 min superfusion of 5 μM chelerythrine. Under these conditions LHRH produced 70.4±1.8 % g_M suppression ($p>0.05$ compared to control). A typical experiment is illustrated in Figure 3-4. The results confirm previous findings that, as for other agonists which suppress g_M, LHRH-mediated suppression is insensitive to PKC inhibitors (Bosma & Hille, 1989; Selyanko *et al.*, 1990; Marrion, 1994; Stemkowski *et al.*, 2002).

Changes in intracellular Ca^{2+} concentrations are known to regulate M-channels. Agonists that suppress g_M mediate an increase in $[Ca^{2+}]_i$, however, it is believed that Ca^{2+} serves only a permissive role and in the case of muscarine is not the signal which mediates agonist-induced g_M suppression (Pfaffinger *et al.*, 1988; Beech *et al.*, 1991; Marrion *et al.*, 1991; Selyanko & Brown, 1996; del Rio *et al.*, 1999; Shapiro *et al.*, 2000; Stemkowski *et al.*, 2002). A limited number of systems do appear to use changes in intracellular Ca^{2+} to mediate agonist-induced g_M suppression. In superior cervical ganglia (SCG) neurons UTP acting via P2Y receptors, and bradykinin acting at its cognate receptor, mediate g_M suppression via Ca^{2+} release from IP_3-sensitye Ca^{2+} stores (Cruzblanca *et al.*, 1998; Bofill-Cardona *et al.*, 2000). The P2Y agonist effect appears to

be limited to mammalian systems, since in BFSG B-neurons ATP acting at P2Y receptors does not meditate g_M suppression via IP_3-induced increases in $[Ca^{2+}]_i$ (Stemkowski *et al.*, 2002).

We next examined whether, like bradykinin, LHRH signals via IP_3 induced alterations in $[Ca^{2+}]_i$. To inhibit IP_3 receptor signaling, cells were dialyzed with 500 μM heparin for 20min. With the 4 MΩ resistance pipettes used, we calculated that after 20-25 min the effective intracellular heparin concentration achieved would equal ~300 μM (Chen *et al.*, 1993; Pusch & Neher, 1988). This well higher than the the IC_{50} for its antagonistic effect at the IP_3 receptor (Ghosh *et al.*, 1988). We found that LHRH-induced suppression of g_M was unaltered, as control suppression at t=20 min was 68.1±1.9 % (n=4) which was not significantly different than the 65.7±2.9 % (n=9) suppression seen at t=20 min with cells dialyzed with heparin (Fig 3-5B). In spite of the lack of effect on g_M suppression, heparin did successfully inhibit g_M over-recovery. Since over-recovery likely results from IP_3-induced release of Ca^{2+} (Chen *et al.*, 1993; Marrion *et al.*, 1991), its blockade indicates that neurons were dialyzed with an effective concentration of heparin. Typical experiments are illustrated in Fig 3-5A. These results suggest that, unlike bradykinin, LHRH does not signal via IP_3.

To determine the role of Ca^{2+} in mediated LHRH-induced g_M suppression, we clamped $[Ca^{2+}]_i$ to either 117 nM with 20 mM BAPTA/8 mM $CaCl_2$ or <10 nM with 20 mM BAPTA/0.4 mM $CaCl_2$, since strong buffering with the fast chelator BAPTA is known to prevent agonist-induced rises in $[Ca^{2+}]_i$ (Beech *et al.*, 1991; Yu *et al.*, 1994). We found that buffering $[Ca^{2+}]_i$ to either 117 nM or <10 nM failed to disrupt LHRH-induced g_M suppression as the % suppression by LHRH was similar among groups dialyzed for 10 min with 4 MΩ pipettes with (a) control pipette (70.5±7.5 % suppression, n=5), (b) 20 mMBAPTA/8 mM Ca^{2+} (75.6±5.9 % suppression, n=6), or (c) 20 mMBAPTA/0.4 mM Ca^{2+} (72.8±4.1 % suppression, n=5) (Fig 3-6B). Typical experiments are illustrated in Fig 3-6A. In spite of its lack of effect on g_M suppression, clamping $[Ca^{2+}]_i$ to a low level did inhibit g_M over-recovery indicating successful dialysis of neurons (Yu, 1995). These results differ from those obtained by (Cruzblanca *et al.*, 1998) which they used to implicate that alterations in $[Ca^{2+}]_i$ meditate the suppression of I_M by bradykinin. Our data suggests that while LHRH-mediated suppression of g_M

involves PLC its effects are not mediated via the 'downstream' pathways involving Ca^{2+} release from IP_3-sensitve Ca^{2+} stores or PKC.

These experiments suggest that while LHRH-mediated suppression of g_M involves PLC, its effects are not mediated via the 'downstream' pathways involving Ca^{2+} release from IP_3-sensitve Ca^{2+} stores or PKC. These results differ from those obtained by Cruzblanca et al. (1998) who found that bradykinin-induced g_M suppression in superior cervical ganglion (SCG) neurons was prevented when $[Ca^{2+}]i$ was clamped to 143 nM. This implicates alterations in $[Ca^{2+}]_i$ in suppression of g_M by bradykinin, at least in mammalian cells. We do not know whether this mechanism is available in BFSG, as bradykinin (1 μM) was without effect on g_M in six cells tested.

Inhibition of PIP_2 resynthesis blocks recovery g_M suppression

PIP_2 is known to modulate TRPM7, HERG, K_{ATP} and Ca^{2+} channels (Hilgemann & Ball, 1996; Fan & Makielski, 1997; Baukrowitz *et al.*, 1998; Huang *et al.*, 1998; Shyng & Nichols, 1998; Bian *et al.*, 2001;Runnels *et al.*, 2002; Wu *et al.*, 2002). Furthermore, PIP_2 increases the activity of M-channels in SCG neurons and KCNQ2/3 channels expressed in oocytes, tsA-201 and COS-1 cells (Zhang *et al.*, 2003, and see Chapter 2). Resynthesis of PIP_2 is also required for the recovery from muscarininc suppression of g_M in both SCG and BFSG neurons and ATP-induced suppression in BFSG (Suh & Hille, 2002, and see Chapter2). These finding suggest that agonist-induced reductions in PIP_2 is the signal responsible for M-channel closure (Suh & Hille, 2002). As LHRH-mediated suppression of g_M did not involve 'downstream' signaling elements, we examined the role of 'upstream' signaling from PLC to see if depletion of PIP_2 from the membrane, and not products of its hydrolysis, promotes closure of M-channels.

At low concentrations wortmannin specifically blocks phosphatidylinositol-3-kinase (PI3K) and at higher micromolar concentrations additionally blocks phosphatidylinositol-4-kinase (PI4K) (Xie *et al.*, 1999; Yano *et al.*, 1993). PI4K is involved in the re-synthesis of PIP_2 in the plasma membrane after agonist-induced PLC-mediated hydrolysis (Fig 1-1). If PLC-induced PIP_2 depletion is required for LHRH-induced g_M suppression, recovery of the response should require PIP_2 re-synthesis. Wortmannin slows g_M recovery by impairing PIP_2 re-synthesis via the inhibition of PI4K

and the co-commitment reduction of PIP (Suh & Hille, 2002; Zhang *et al.*, 2003), and see Chapter 2). Treatment of BFSG B-neurons with 10 μM wortmannin for 5 min slowed the recovery phase of the LHRH response so that 4.5 min after suppression, only 42.5±8.2 % (n=8) of the pre-LHRH g_M remained (Fig 3-7A and 3-8). Treating neurons similarly with 1 μM wortmannin or the specific PI3K inhibitor LY294002 (10 μM) to cause inhibition of PI3K, but not PI4K, reduced the recovery phase significantly less than 10μM wortmannin so that 86.1±5.4 % (n=6; $p<0.01$) (Fig 3-7B and 3-8) or 95.1±4. 8% (n=6; $p<0.01$) (Fig 3-7C and 3-8) of the pre-LHRH g_M remained.

Suh and Hille (2002) have shown that non-hydrolysable ATP analogues act similarly to wortmannin in slowing the recovery of muscarine-induced M-channel suppression. Removing a source of phosphate donors from the lipid and inositol phosphate cycles is comparable to inhibition of PI4K in that both prevent the resynthesis of PIP_2 following PLC-mediated hydrolysis. We tested whether this was true for transduction of the LHRH signal. Cells dialysed for 10 min with control internal solution containing 2mM ATP in a glucose-free external solution repeatedly challenged with LHRH showed strong suppression and recovery of g_M (Fig 3-9A). Sixty seconds post the initial LHRH application, g_M had over-recovered to 117.3±4.2 % (n=7; Fig 3-10) of the initial current. Dialysis of cells in a similar manner with an ATP-free internal did not cause any significant changes in the recovery following suppression (101.9±8.2% recovery vs. pre-LHRH (n=6; $p>0.05$; Fig 3-9B and 3-10). Dialysis of neurons with an internal solution containing 0 mM ATP and 3 mM of the non-hydrolysable ATP analogue, AMP-PNP (adenosine 5'-(β,γ-imido)triphosphate tetralithium salt hydrate) did not affect the initial suppression of g_M but strongly reduced the recovery, so that only 59±7.9 % of the initial current remained 60 sec after the initial LHRH-induced suppression (n=9; $P<0.001$ vs. both 2mM ATP and 0mM ATP) (Fig 3-9C and 3-10). Subsequent LHRH applications eventually led to a near complete ablation of resting g_M (Fig 3-9C). This suggests that hydrolysable ATP is required for LHRH-induced g_M recovery. The above results fit within our hypothesis that ATP is required as a phosphate donor in PI4K mediated reaction to allow for the resynthesis of PIP_2, and that inhibiting the resynthesis of PIP_2 following LHRH-suppression prevents the recovery of g_M. This and the above data indicate that the mechanism of LHRH-induced M-channel

suppression occurs via a loss of plasma membrane PIP_2 following PLC-mediated hydrolysis.

Anti-PIP_2 Antibodies Reduce Basal g_M and LHRH-Mediated Suppression

Well–characterized anti-PIP_2 neutralizing antibodies have been developed which reduce PIP_2-dependent channel activity in a variety of cell types (Huang *et al.*, 1998; Liou *et al.*, 1999; Zhang *et al.*, 1999; Bian *et al.*, 2001; Chuang *et al.*, 2001; Runnels *et al.*, 2002; Zhang *et al.*, 2003). Dialysis of neurons for 25 min with anti-PIP_2 antibody (1:100) caused g_M to run-down to 56.4±11.2 % (n=6) of initial levels (Fig 3-12A). This was significantly more than horse serum (1:100) dialyzed controls which ran-down to 94.6±7.9 % (n=8) of initial levels after 25 min (Fig 3-12A). In addition to the effect on resting M-channel activity, PIP_2 antibodies also reduced the LHRH-induced suppression of g_M so that after 25 min of dialysis, LHRH-induced suppression was only 64.7±5.5 % (n=6) of initial levels (Fig 3-12B). This was significantly less than horse serum controls for which LHRH-induced suppression was maintained at 86.6±8.1 % (n=6) of initial values (Fig 3-12B). Typical experiments are illustrated in Figs 3-11A and B. We interpret these results to suggest that by neutralizing PIP_2 with the addition of the antibody we inhibit both the PIP_2-PLC interaction and the PIP_2-M-channel interaction.

DISCUSSION

Two different, agonist specific, mechanisms have been proposed to explain how receptors that couple to G_q can promote g_M suppression. Available evidence from rat SCG neurons are consistent with the hypothesis that both bradykinin and ATP suppress g_M via the IP_3-mediated release of Ca^{2+} from intracellular stores (Bofill-Cardona *et al.*, 2000; Cruzblanca *et al.*, 1998). By contrast, data from frog and rat sympathetic neurons and from KCNQ2/3 channels in expression systems are consistent with the involvement of 'upstream' signaling in the action of muscarine. This would involve the PLC-mediated depletion of PIP_2 (see Chapter 2, and Suh & Hille, 2002; Zhang *et al.*, 2003)

The results presented here are consistent with the hypothesis that the mechanism of LHRH-induced M-channel suppression also proceeds via the depletion of PIP_2 (see Chapter 2). This mechanism may also contribute to P2Y-induced g_M suppression in these

neurons (see Chapter 2). In summary, LHRH mediates suppression of g_M via PLC (Fig 3-1) but not via the products of PIP_2 hydrolysis (Fig 3-4, 3-5, 3-6). Recovery from LHRH-induced suppression is dependent upon proper functioning of the lipid and inositol phosphate cycles. Inhibition of PIP_2 resynthesis by inhibiting PI4K by wortmannin (10 μM) slows recovery of g_M following LHRH application (Fig 3-7A). The non-hydrolysable ATP analogue AMP-PNP mimics this effect (Fig 3-9C), suggesting that available ATP is required to 'fuel' the resynthesis of PIP_2 in phosphate-dependent reaction following LHRH-induced hydrolysis. Inhibition of PI3K, which is not involved in PIP_2 resynthesis, by either wortmannin (1 μM) or LY294002 (10 μM) does not slow the rate of recovery of g_M following LHRH application (Fig 3-7B and 3-7C). PIP_2 neutralizing antibodies reduce both the basal level of g_M and the ability of LHRH to suppress g_M (Fig 3-11). All of these results are best explained within the PIP_2 depletion hypothesis. This states that M-channels are maintained in an open configuration by the presence of plasma membrane PIP_2, and LHRH-induced activation of PLC mediates the hydrolysis of PIP_2. The result is a loss of plasma membrane PIP_2 thus promoting M-channels to assume a closed configuration (i.e., g_M suppression).

The activation of PLC by LHRH is supported here by the fact that the PLC inhibitor U73122, but not the inactive isomer, U73343 antagonized LHRH-induced g_M suppression (Fig 3-1). This supports previous evidence that likely all agonists which mediate M-channel suppression do so by signaling via PLC in a $G_{q/11}$ dependent (see Chapter 2 Caulfield *et al.*, 1994; Marrion, 1997; Cruzblanca *et al.*, 1998; Haley *et al.*, 1998; Pfaffinger *et al.*, 1988; del Rio *et al.*, 1999; Haley *et al.*, 2000b; Haley *et al.*, 2000a; Guo & Schofield, 2002; Stemkowski *et al.*, 2002; Suh & Hille, 2002).

Previous data has suggested that muscarinic signaling while being dependent upon PLC, does not involve downstream products (IP_3, Ca^{2+}, or PKC) (Bosma & Hille, 1989; Beech *et al.*, 1991; Marrion *et al.*, 1991; Yu *et al.*, 1994; Yu, 1995; Selyanko & Brown, 1996; del Rio *et al.*, 1999; Shapiro *et al.*, 2000; Stemkowski *et al.*, 2002). This is also true of the LHRH receptor, as blocking PKC, or Ca^{2+} signaling from IP_3-sensitve stores did not antagonize LHRH-mediated g_M suppression (Figs 3-4, 3-5, 3-6). This contrasts with bradykinin and P2Y receptors in SCG neurons, which may mediate M-channel suppression via Ca^{2+} release from IP_3-sensitve stores (Bofill-Cardona *et al.*,

2000; Cruzblanca *et al.*, 1998). Bradykinin did not suppress g_M in BFSG neurons. Whether this lack of effect simply reflects an absence of functional bradykinin receptors or their failure to properly signal via the IP_3/Ca^{2+} to cause M-channel closure remains to be determined.

Maintenance (the prevention of run-down) of g_M requires ATP or metabolic substrates such as glucose or pyruvate (Pfaffinger, 1988; Pfaffinger *et al.*, 1988; Simmons *et al.*, 1990; Tokimasa & Akasu, 1990; Chen & Smith, 1992; Simmons & Schneider, 1998; Suh & Hille, 2002). The recovery from muscarine-induced inhibition requires ATP yet the addition of glucose or pyruvate will not suffice (Suh & Hille, 2002). This suggests the process requires a higher level of phosphorylation than that required to prevent rundown, implying that rundown and agonist-induced suppression involve similar processes. This concept is supported by the fact that long term inhibition of PI4K by high concentrations of wortmannin accelerates rundown (Tokimasa *et al.*, 1995; Suh & Hille, 2002; Zhang *et al.*, 2003). Both wortmannin and the lack of hydrolysable ATP cause progressive attenuation of g_M recovery after successive applications of agonist. These findings are easily explainable within the lipid kinase and PI-polyphosphate hypothesis in that ATP and PIP_2 synthesis may be required for recovery. A green fluorescent protein-tagged pleckstrin homology domain of PLC-δ (GFP-PH) reporter has recently been used to monitor PIP_2 hydrolysis and its relative membrane level in M_1-receptor transfected CHO cells (Zhang *et al.*, 2003). It was found that acetylcholine (ACh) promoted a rapid loss of PIP_2 from the membrane which was rapidly reversible upon agonist wash out. Wortmannin at 10 μM, but not at 1 μM, strongly slowed the rate of recovery of membrane PIP_2 levels (Zhang *et al.*, 2003). This supports the hypothesis that the delaying of recovery of M-channels following ACh-induced suppression by wortmannin is due to a prevention of the resynthesis of plasma membrane PIP_2 (Zhang *et al.*, 2003). In this chapter, it is demonstrated that the recovery from LHRH-induced g_M suppression is also sensitive to high concentrations of wortmannin and non-hydrolysable ATP analogues (Figs 3-7 and 3-9), suggesting that LHRH-induced g_M suppression in BFSG proceeds in a similar manner to that of muscarine and ATP (Suh & Hille, 2002; Zhang *et al.*, 2003, see Chapter 2)

PIP_2 antibodies inhibited the steady-state g_M (Fig 3-11 and 3-12). This observation is consistent with the findings of others with KCNQ2/3 channels and is furthermore consistent with the hypothesis that PIP_2 removal invokes channel closure (Zhang *et al.*, 2003). This effect has been previously reported for other ionic conductances in a variety of cell types (Huang *et al.*, 1998; Liou *et al.*, 1999; Bian *et al.*, 2001; Chuang *et al.*, 2001). PIP_2 antibodies also attenuated the effect of LHRH on g_M suppression (Fig 3-11 and 3-12). This is similar to the effect on muscarine and ATP reported in Chapter 2. However, in experiments described within that chapter, antibodies appeared not to affect the steady-state g_M. This led to the conclusion that the antibodies protected PIP_2 from the action of PLC whilst not affecting the PIP_2-M-channel interaction. The reason for the difference reported here is not yet clear. Possible causes may be increased avidity of the antibody towards PIP_2 or increased effective antibody concentration in the current formulation as supplied. Nevertheless the antibody results are consistent with the hypothesis that removal of PIP_2 invokes channel closure. Thus, agonist-induced PIP_2 removal may invoke channel closure.

Conclusion

In summary, the data present here support the hypothesis that the mechanism of LHRH-induced g_M suppression in BFSG, like ATP and muscarine involves the PLC-mediated depletion of PIP_2. This effect of LHRH is then unlike that of the other peptide neurotransmitter, bradykinin, as LHRH-mediated suppression of g_M does not involve Ca^{2+} release from IP_3-sensitive stores.

Figure 3-1

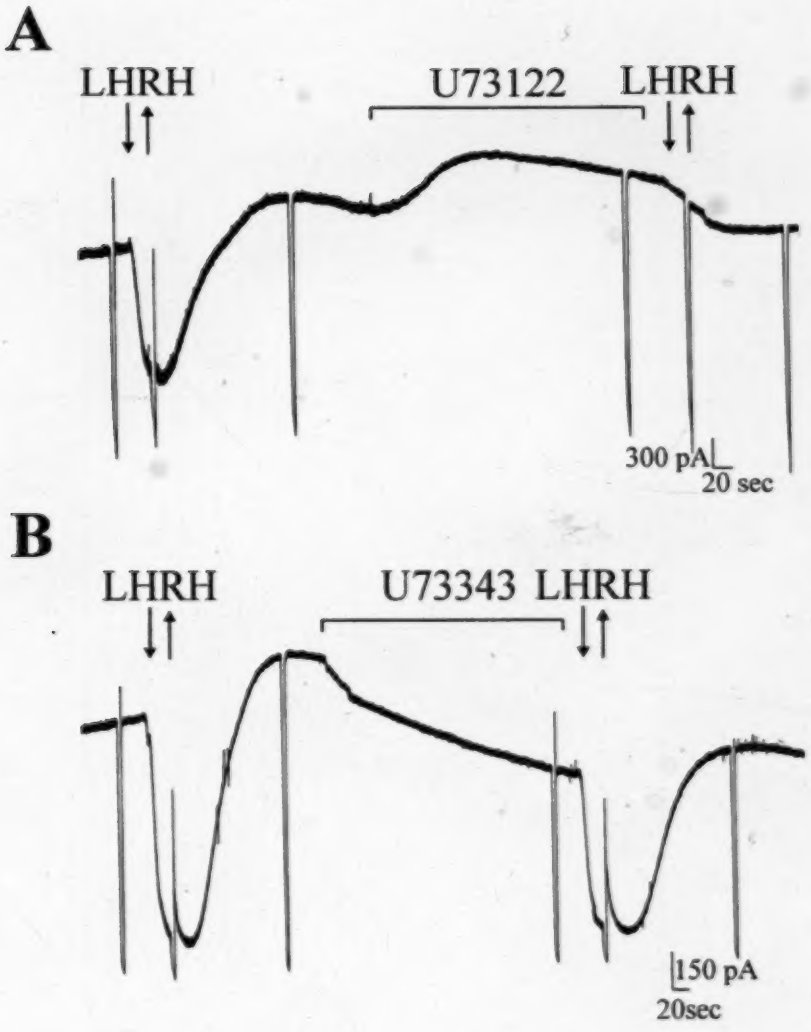

A
LHRH
U73122
LHRH
300 pA
20 sec
B
LHRH
U73343
LHRH
150 pA
20sec

Figure 3-2. Current-voltage plots derived from 4.5sec ramp commands to measure membrane conductance, as explained in the methods section. Suppression of current positive to -75 mV (i.e. in the M-conductance (g_M) range) by LHRH. Washout of LHRH was associated with I_M over-recovery.

Figure 3-2

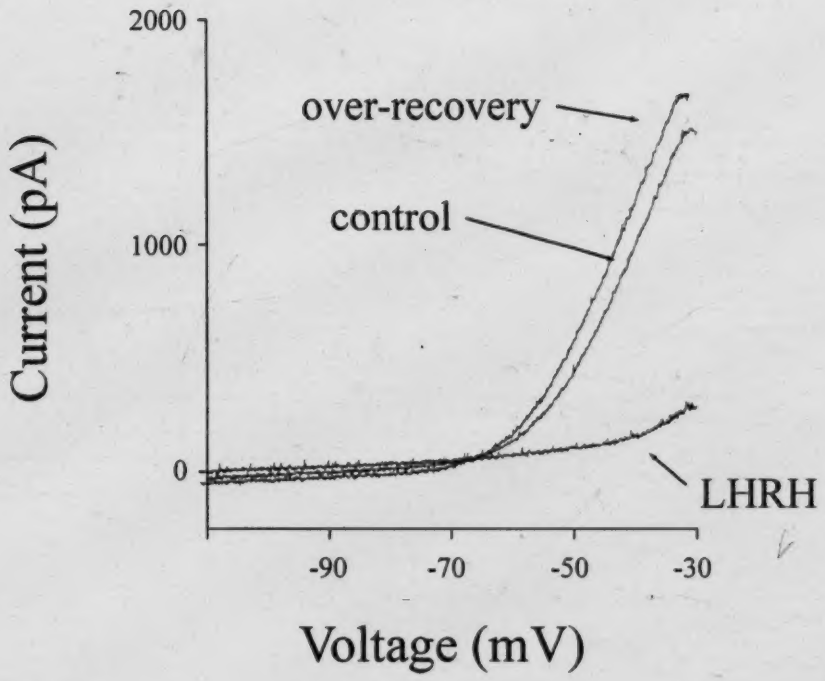

2000
1000
0
Current (pA)
over-recovery
control
LHRH
-90
-70
-50
-30
Voltage (mV)

Figure 3-3. Summary data of the effect of U73122 (10 μM) on blocking LHRH-induced I_M suppression. Lack of effect of the inactive analogue U73343 (10 μM) on LHRH-induced I_M suppression. I_M suppression by LHRH was compared in the presence of drug to that prior to drug application.

Figure 3-3

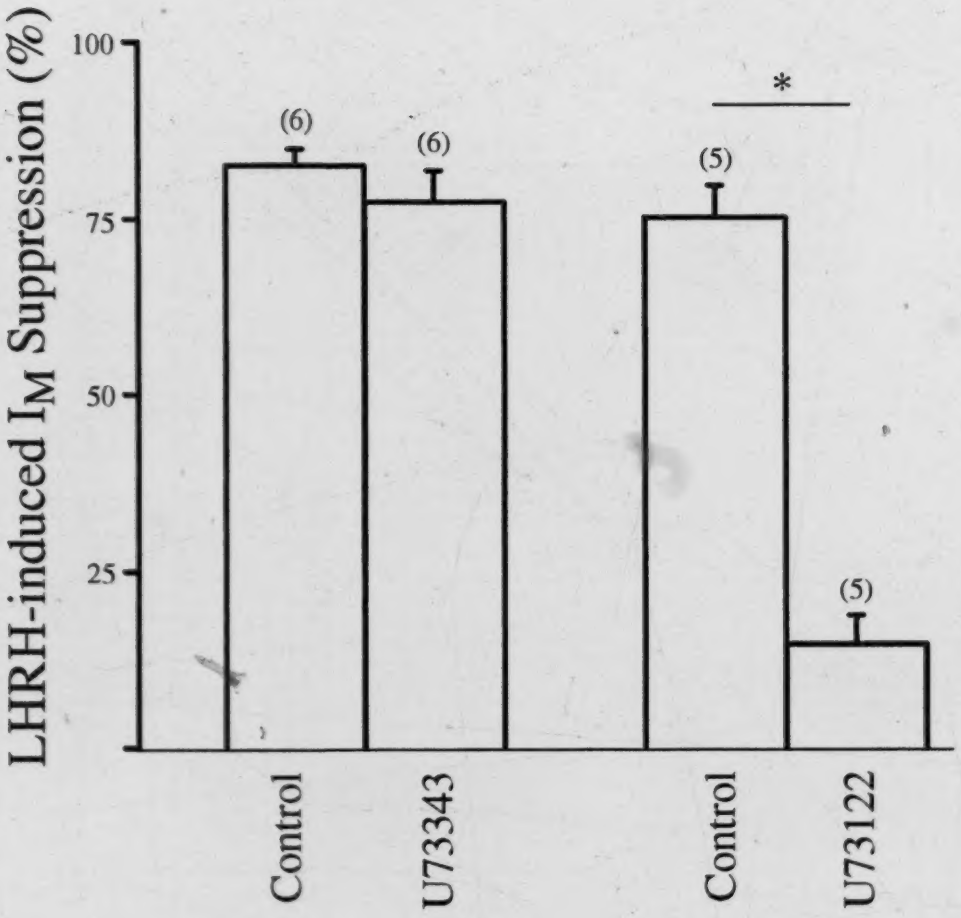

LHRH-induced I_M Suppression (%)
100
75
50
25
(6)
(6)
(5)
(5)
*
Control
U73343
Control
U73122

Figure 3-4. Lack of effect of chelerythrine on LHRH-induced M-current suppression. **(A)** Chart recording of steady-state I_M at -30 mV in BFSG B-neurons. Downward deflections are responses to voltage ramps (from -30 mV to -110 mV) used to assess membrane conductance (voltage records omitted for clarity). Pipette contained 10 μM chelerythrine and 5 μM chelerythrine was applied extracellularly as indicated by the bar. Note the similar effect of LHRH on I_M initially as compared to after 10 min after gaining whole cell access and initiating recording. **(B)** Summary data illustrating the lack of effect of chelerythrine on LHRH-induced I_M suppression.

Figure 3-4

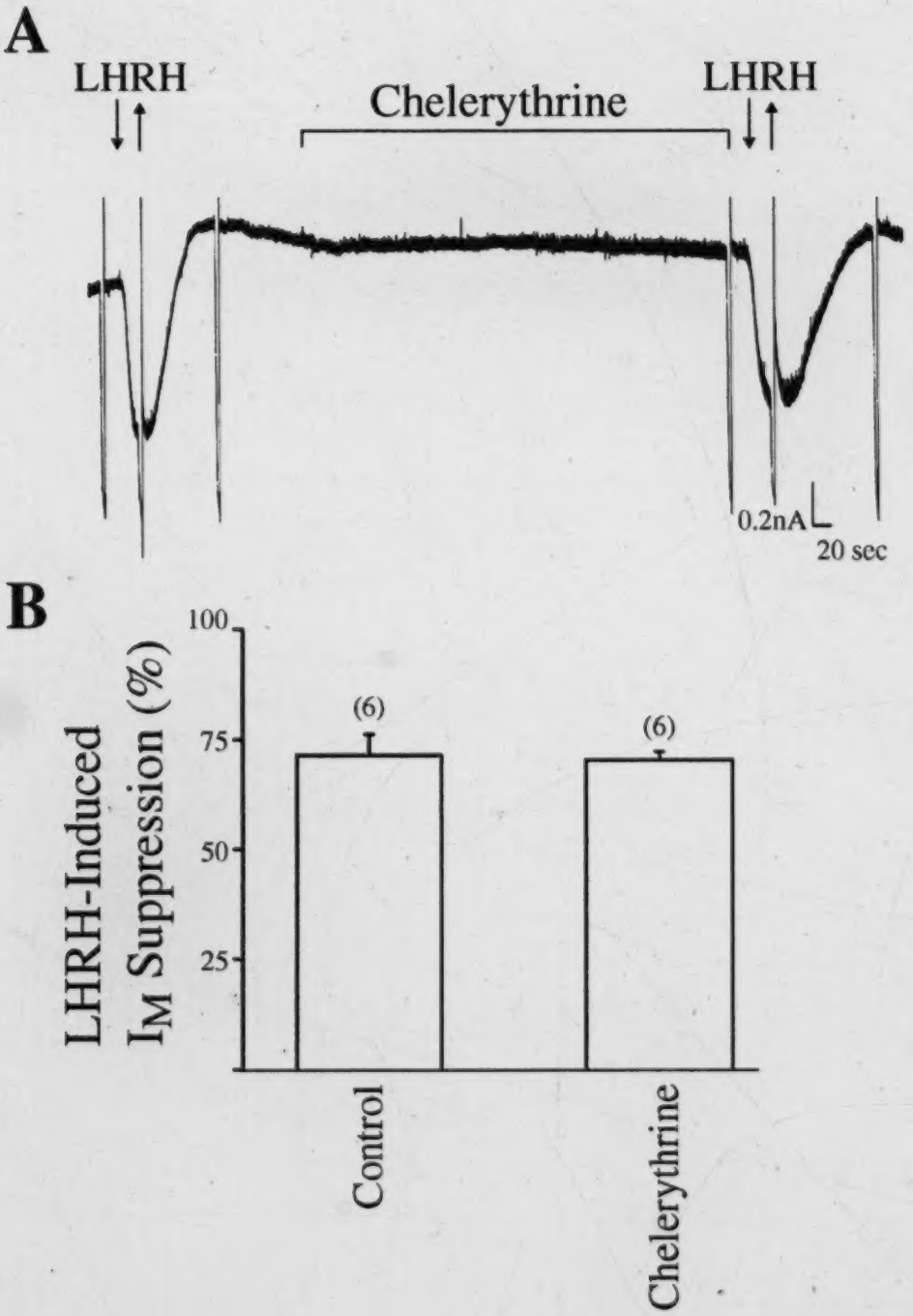

A
LHRH
Chelerythrine
LHRH
0.2nA
20 sec
B
LHRH-Induced
I_M Suppression (%)
100
75
50
25
(6)
(6)
Control
Chelerythrine

Figure 3-5. Lack of effect of intracellular heparin on LHRH-induced M-current suppression. **(A)** Chart recording of steady-state I_M at -30 mV in BFSG B-neurons. Downward deflections are responses to voltage ramps (from -30 mV to -110 mV) used to assess membrane conductance (voltage records omitted for clarity). Dialysis of B-neurons with control internal for 20 min with 500 μM heparin does not alter LHRH-induced suppression compared to cells dialyzed for 20 min with control internal. **(B)** Summary data illustrating the lack of effect of heparin on LHRH-induced I_M suppression.

Figure 3-5

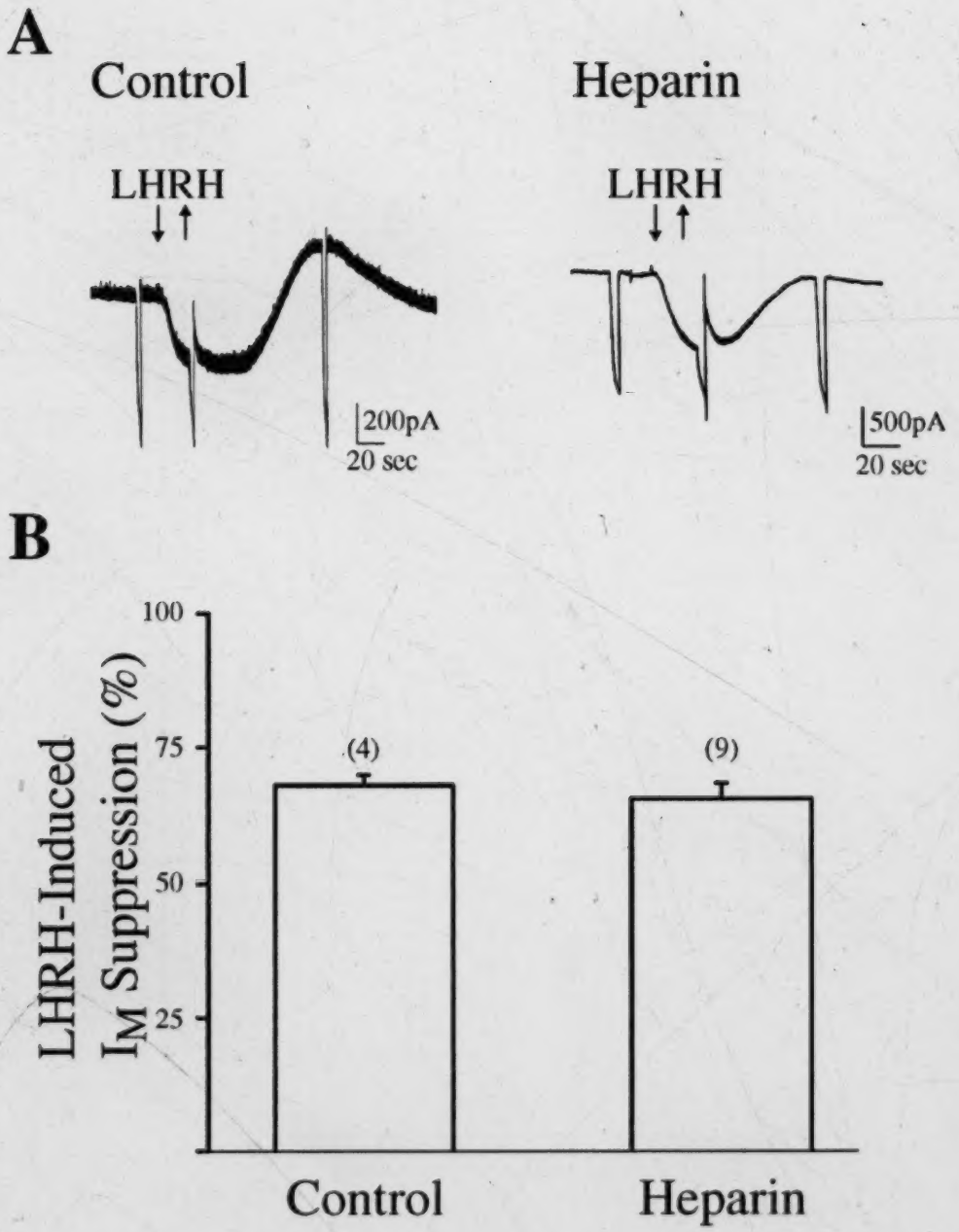

A
Control
Heparin
LHRH
LHRH
200pA
20 sec
500pA
20 sec
B
LHRH-Induced
I_M Suppression (%)
100
75
50
25
(4)
(9)
Control
Heparin

Figure 3-6. Lack of effect of intracellular BAPTA on LHRH-induced M-current suppression. **(A)** Chart recording of steady-state I_M at -30 mV in BFSG B-neurons. Downward deflections are responses to voltage ramps (from -30 mV to -110 mV) used to assess membrane conductance (voltage records omitted for clarity). Dialysis of B-neurons with internal containing 20 mM BAPTA + 8 mM Ca^{2+} or internal containing 20 mM BAPTA + 0.4 mM Ca^{2+} for 10 min does not alter LHRH-induced I_M suppression compared to B-cells dialyzed for 10 min with control internal. **(B)** Summary data illustrating the lack of effect of intracellular BAPTA on LHRH-induced I_M suppression.

Figure 3-6

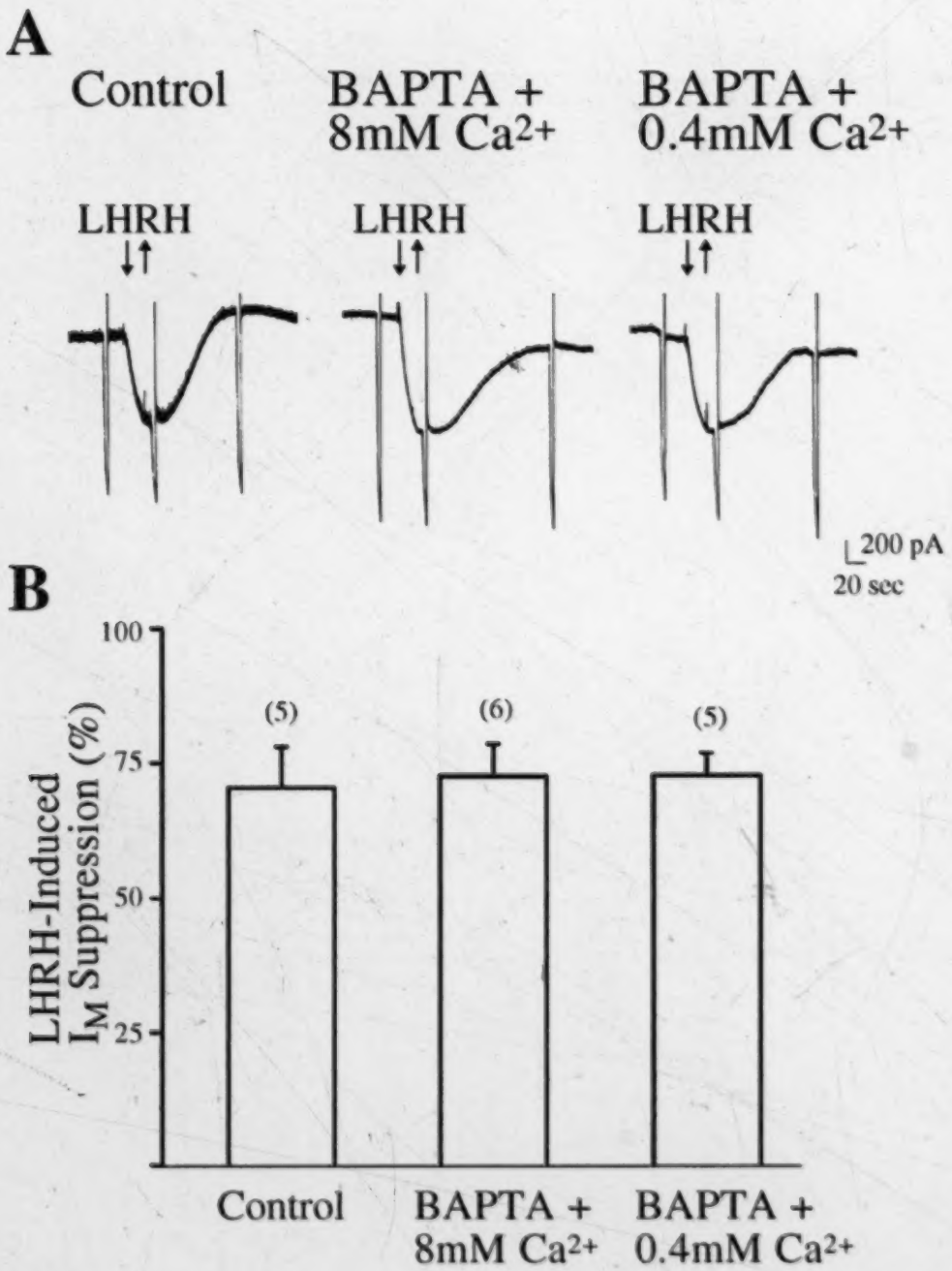

A
Control
BAPTA +
8mM Ca2+
BAPTA +
0.4mM Ca2+
LHRH
LHRH
LHRH
200 pA
20 sec
B
LHRH-Induced
IM Suppression (%)
100
75
50
25
(5)
(6)
(5)
Control
BAPTA +
8mM Ca2+
BAPTA +
0.4mM Ca2+

Figure 3-7. Effects of PI3-kinase and PI4-kinase inhibitors on recovery from LHRH-induced M-current suppression. Chart recording of steady-state I_M at -30 mV in BFSG B-neurons. Downward deflections are responses to voltage ramps (from -30 mV to -110 mV) used to assess membrane conductance (voltage records omitted for clarity). **(A)** Slowing of the rate of recovery of LHRH response in the presence of 10μM wortmannin to inhibit PI4-kinase and PI3-kinase. **(B)** Lack of effect of inhibition of PI3-kinase on LHRH responses by 1 μM wortmannin. **(C)** Lack of effect of the PI3-kinase inhibitor LY294002 (10 μM) on LHRH responses.

Figure 3-7

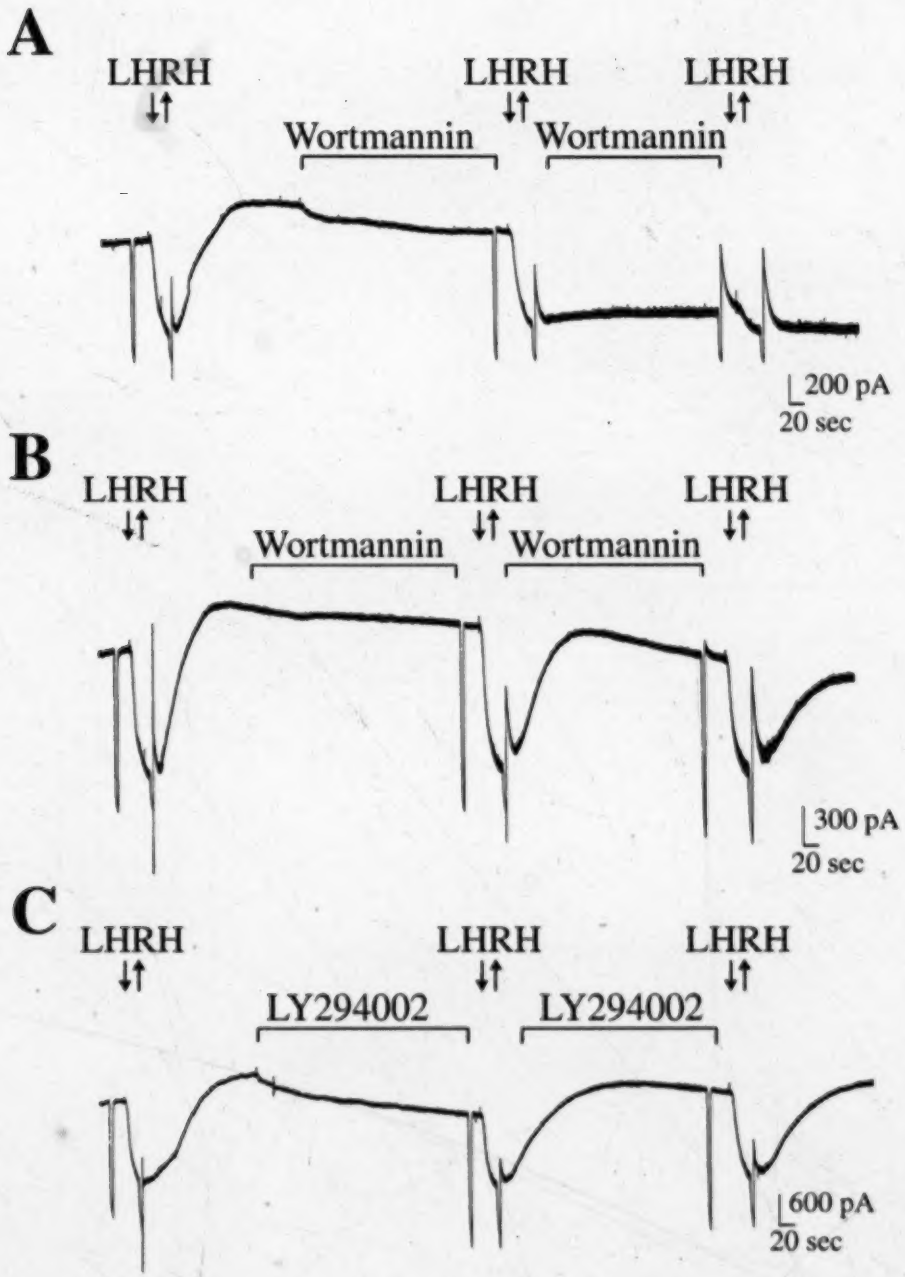

A
LHRH
LHRH
LHRH
Wortmannin
Wortmannin
200 pA
20 sec
B
LHRH
LHRH
LHRH
Wortmannin
Wortmannin
300 pA
20 sec
C
LHRH
LHRH
LHRH
LY294002
LY294002
600 pA
20 sec

Figure 3-8. Summary data from groups of cells as illustrated in figure 3-7. The % recovery is I_M in the presence of drug compared 4.5 min after LHRH application I_M recorded pre LHRH application. Inhibition of PI4K by 10 μM wortmannin significantly reduces the % recovery of I_M compared to inhibition of PI3K by 1 μM wortmannin or 10 μM LY294002.

Figure 3-8

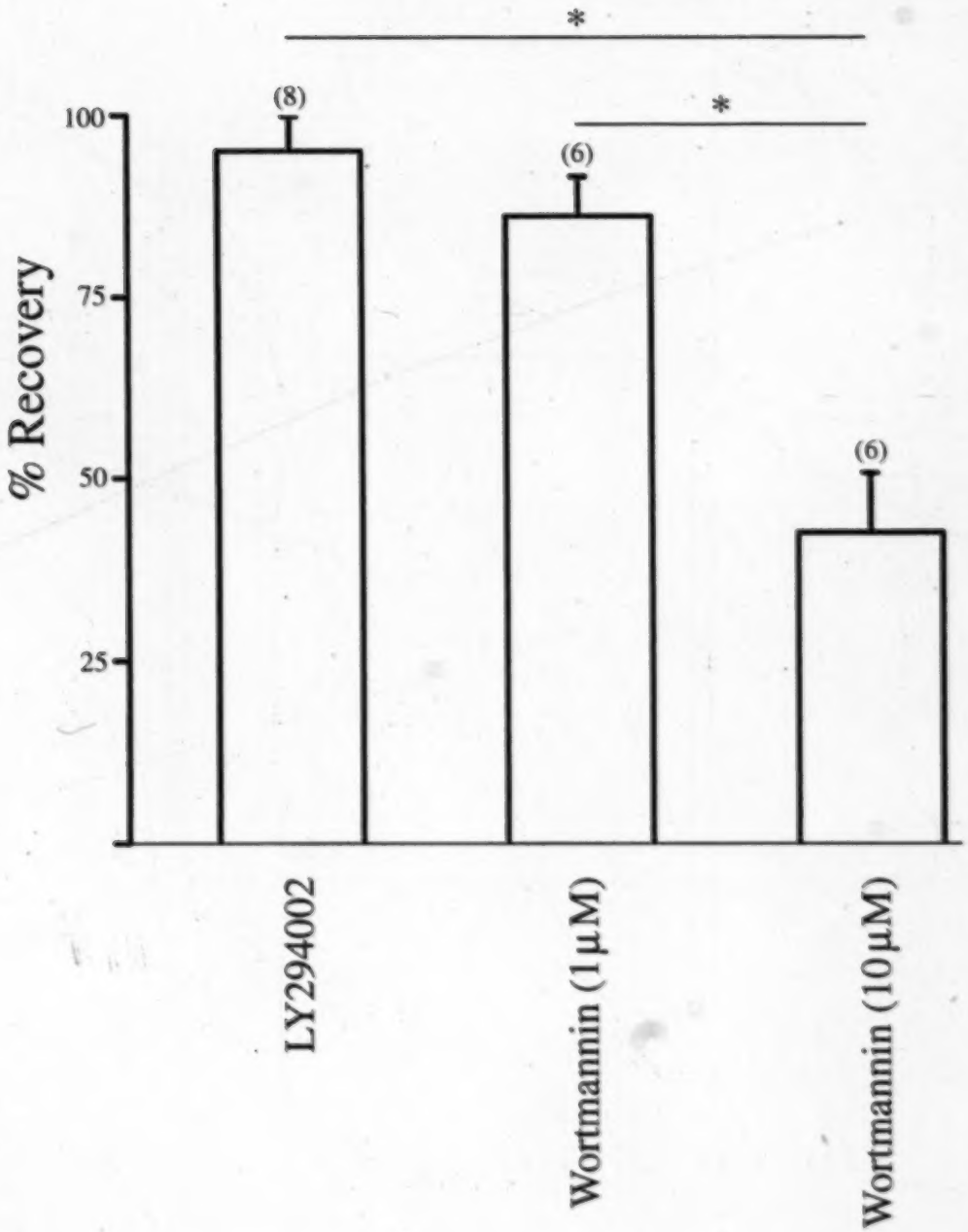

*
*
(8)
(6)
(6)
100
75
50
25
% Recovery
LY294002
Wortmannin (1 µM)
Wortmannin (10 µM)

Figure 3-9. Non-hydrolyzable ATP analogues slow recovery of LHRH-mediated M-current suppression. Chart recording of steady-state I_M at -30 mV in BFSG B-neurons. Downward deflections are responses to voltage ramps (from -30 mV to -110 mV) used to assess membrane conductance (voltage records omitted for clarity). Effect of three successive applications of 100nM LHRH on I_M with cells dialyzed with pipettes containing **(A)** 2 mM ATP, **(B)** 0 mM ATP + 0 mM AMP-PNP, and **(C)** 3 mM AMP-PNP to test requirement of ATP for LHRH-induced I_M recovery. All recordings were taken 10 min after gaining whole cell access.

Figure 3-9

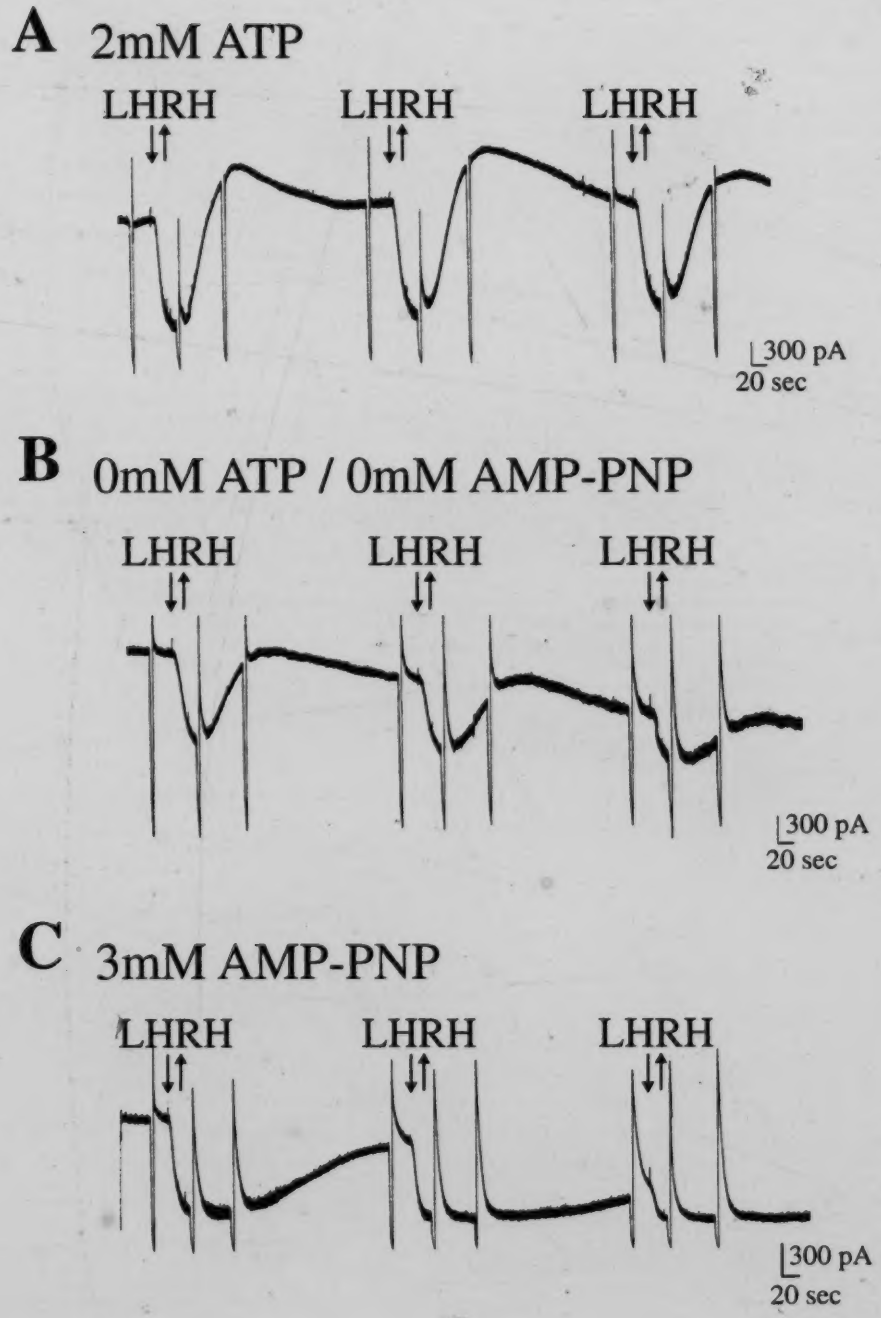

A
2mM ATP
LHRH
LHRH
LHRH
300 pA
20 sec
B
0mM ATP / 0mM AMP-PNP
LHRH
LHRH
LHRH
300 pA
20 sec
C
3mM AMP-PNP
LHRH
LHRH
LHRH
300 pA
20 sec

Figure 3-10. Summary data from data from groups of cells as illustrated in figure 3-9. The % recovery is I_M recorded 1 min post LHRH application compared to I_M recorded pre-LHRH application. Cells dialyzed with pipettes containing 3 mM AMP-PNP/0 mM ATP had significantly reduced % recovery compared to cells dialyzed with pipettes containing 0 mM AMP-PNP/0 mM ATP or 0 mM AMP-PNP/2 mM ATP.

Figure 3-10

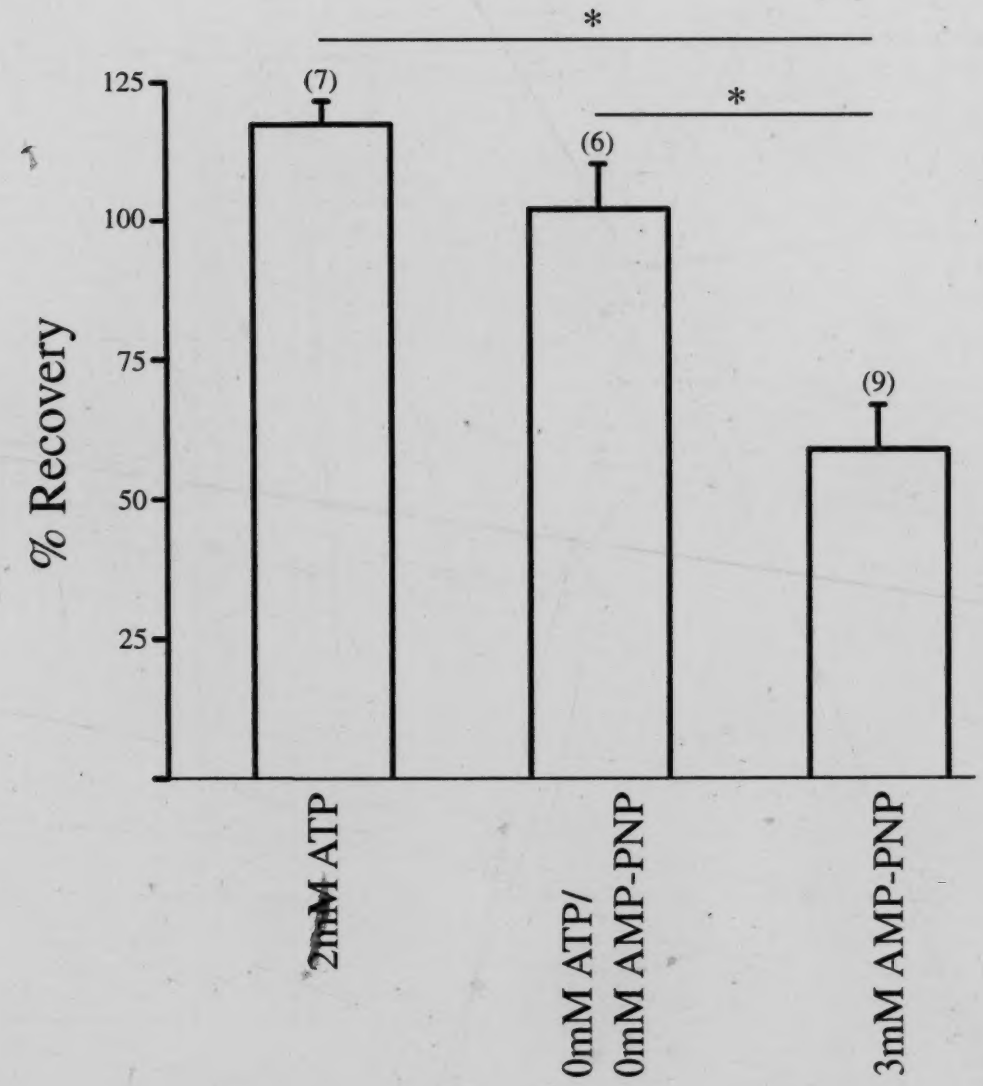

*
*
125
100
75
50
25
% Recovery
(7)
(6)
(9)
2mM ATP
0mM ATP/
0mM AMP-PNP
3mM AMP-PNP

Figure 3-11. PIP_2 neutralizing antibodies reduce steady state M-current and LHRH-mediated suppression. Chart recording of steady-state I_M at -30 mV in BFSG B-neurons. Downward deflections are responses to voltage ramps (from -30 mV to -110 mV) used to assess membrane conductance (voltage records omitted for clarity). Left hand panel shows responses recorded 5 min after establishing whole cell access, while the right hand panels show the responses recorded after 25 min of recording. **(A)** Dialyzing B-neurons with a pipette containing 1:100 anti-PIP_2 antibodies produced a reduction in steady state I_M as well as a reduction in LHRH-mediated suppression. **(B)** Lack of effect 1:100 horse serum on I_M or LHRH-mediated suppression.

Figure 3-11

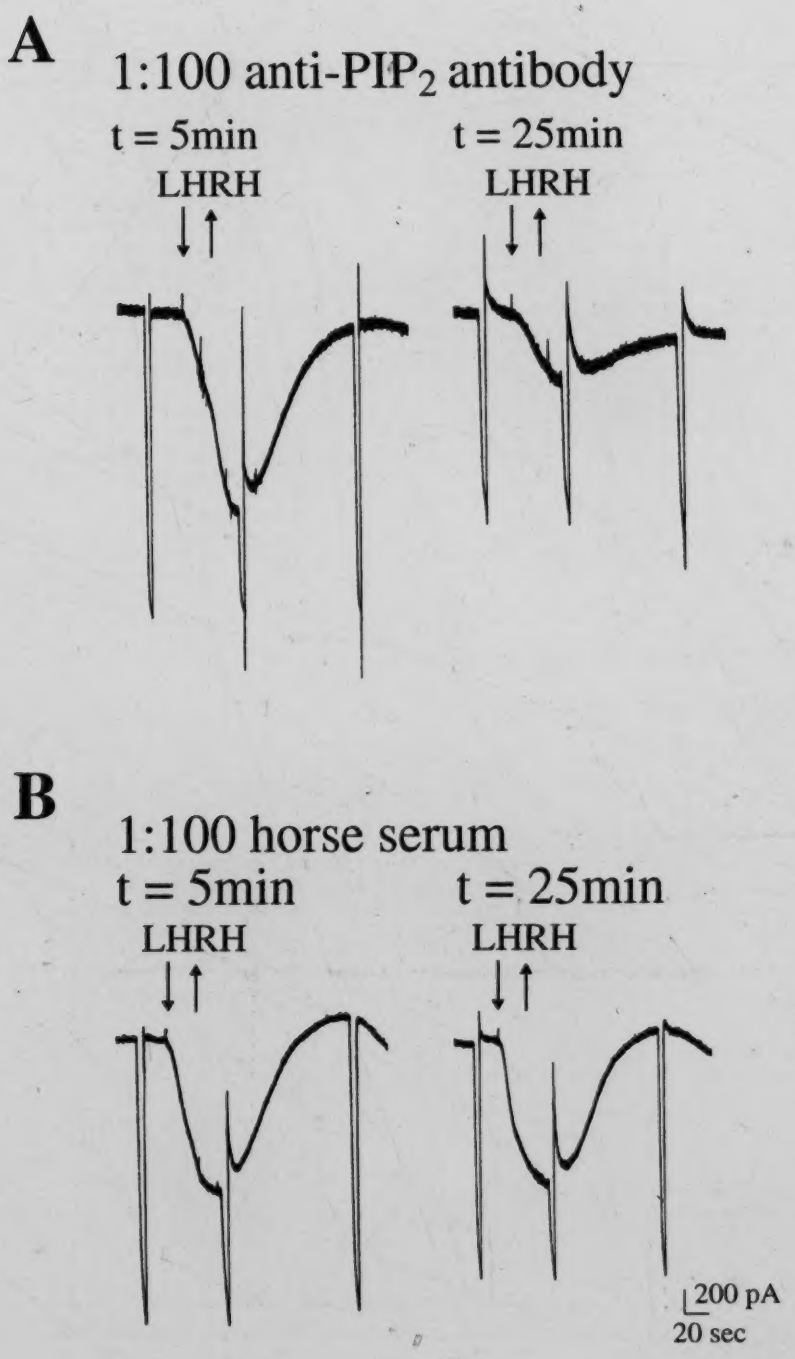
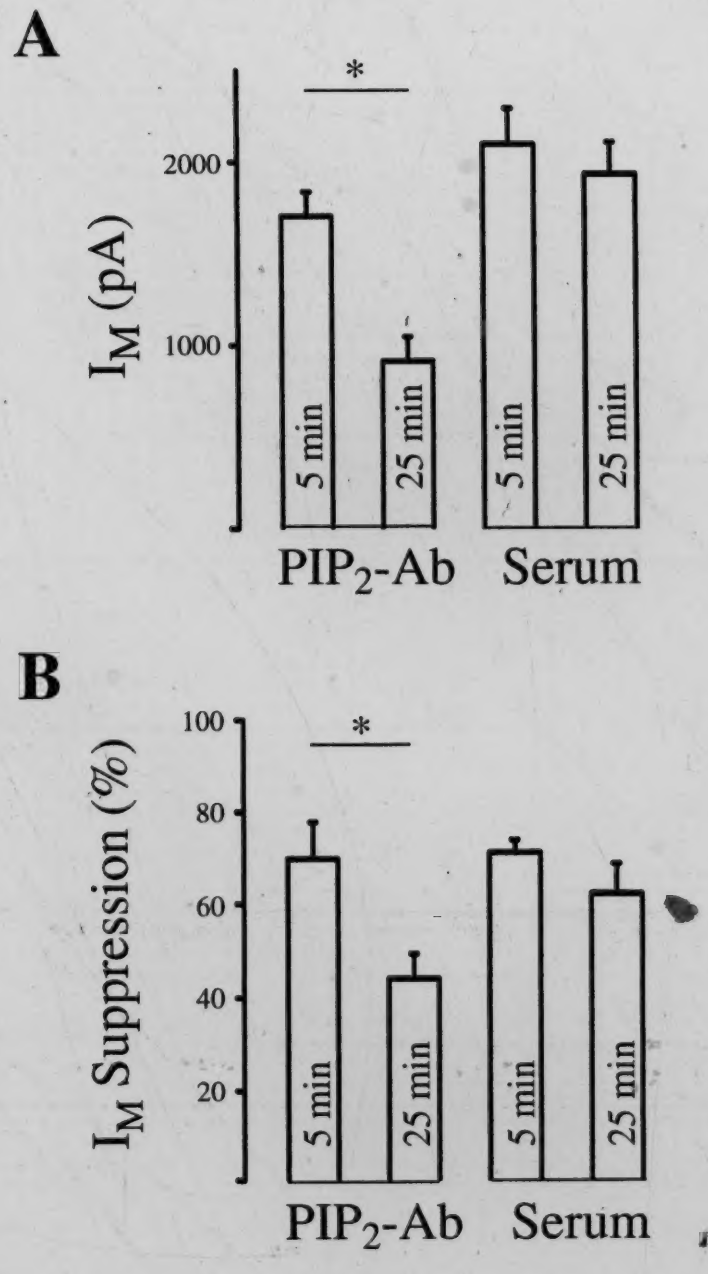

A
1:100 anti-PIP2 antibody
t = 5min
LHRH
t = 25min
LHRH
B
1:100 horse serum
t = 5min
LHRH
t = 25min
LHRH
200 pA
20 sec

Figure 3-12. Summary data from groups of cells as illustrated in figure 3-11. **(A)** Dialyzing cells for 25 min with anti-PIP_2 antibodies significantly reduced steady state I_M, an effect which was not seen by dialyzing cells with an equal amount of horse serum. **(B)** Dialyzing cells for 25 min with anti-PIP_2 antibodies significantly reduced LHRH-induced I_M suppression, an effect which was not seen by dialyzing cells with an equal amount of horse serum.

Figure 3-12


A
*
2000
1000
I_M (pA)
5 min
25 min
5 min
25 min
PIP_2-Ab
Serum
B
*
100
80
60
40
20
I_M Suppression (%)
5 min
25 min
5 min
25 min
PIP_2-Ab
Serum

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Chapter 4

Neurotrophic Regulation of Calcium Channels by the Peptide Neurotransmitter Luteinizing Hormone Releasing Hormone

Some of the results from this chapter have appeared in the Journal of Neuroscience (2003); 23 (18): 7169-7175

INTRODUCTION

The neuromodulatory effects of neuropeptides which act through G-protein coupled receptors (GPCR) and which persist for periods of several minutes are very well documented (Ikeda & Dunlap, 1999, and see Chapter 1 and 3). Although neuropeptides also exert longer term effects which may alter the properties of neurons over periods of days or weeks (McKeon & Zigmond, 1993; Mohney & Zigmond, 1998) comparatively little is known about the cellular mechanisms and physiological consequences of these neurotrophic actions.

As discussed in the *General Introduction*, B-cells of BFSG receive synaptic input from preganglionic B-fibers whereas the smaller C-cells, receive synaptic input from preganglionic C-fibers (Horn *et al.*, 1988; Ivanoff & Smith, 1995; Jobling & Horn, 1996; Thorne & Horn, 1997). The peptide luteinizing hormone releasing hormone (chicken II-gonadotropin-releasing hormone) (LHRH) is released exclusively from preganglionic C-fibers, inducting a late-slow excitatory postsynaptic potential in post-synaptic B- and C- neurons (Jan *et al.*, 1979; Jan *et al.*, 1980b; Jan *et al.*, 1980a; Adams *et al.*, 1982; Jobling & Horn, 1996; Jones, 1987; Thorne & Horn, 1997; Troskie *et al.*, 1997). The latter response reflects the diffusion of the peptide from preganglionic C-fibers to receptors on B-cells (Jan *et al.*, 1979; Jan *et al.*, 1980b; Jobling & Horn, 1996; Thorne & Horn, 1997). Because LHRH is tonically released from C-fibers *in vivo*, it may mediate an ongoing physiological interaction between the vasomotor C-fiber and the exocrine B-cell system (Ivanoff & Smith, 1995; Ford *et al.*, 2000).

LHRH has also been shown to stimulate mitogen activated protein kinase (p42/44MAPK; ERK1/2) activity in mammalian gonadotropes (Haisenleder *et al.*, 1998), αT3-1 gonadotrope cells (Sim *et al.*, 1995; Sundaresan *et al.*, 1996; Reiss *et al.*, 1997) and rat GnRH-receptor transfected GH_3 cells (Han & Conn, 1999). Since the properties and number of Ca^{2+} channels in BFSG appear to be maintained by nerve growth factor (NGF) (Lei *et al.*, 1997; Petrov *et al.*, 2001) and this also involves activation of Ras and MAPK (Lei *et al.*, 1998), we tested whether LHRH is capable of regulating Ca^{2+} channels in BFSG via this pathway.

These experiments were feasible because individual sympathetic neurons isolated from adult bullfrogs remain viable for several weeks in the absence of supplementary

neurotrophins, in a serum-free, low cellular density, defined medium, 'neuron enriched' culture (Lei *et al.*, 1997). This system is free of potential trophic effects of serum, glial cells (Assouline *et al.*, 1987), fibroblasts (Unsicker *et al.*, 1987) or target tissues (Horackova *et al.*, 1996). It therefore provides a unique opportunity to study potential neurotrophic actions of neurotransmitters without complicating effects of exogenously applied or endogenous neurotrophins. It was found that exposure of cultured neurons to LHRH promoted a significant increase in the expression of functional N-type Ca^{2+} channels in BFSG B-cells, and that this regulation was attenuated following impairment of the Ras-ERK pathway. Moreover, removal of all sources of ganglionic LHRH *in vivo* resulted in decreased functional expression of Ca^{2+} channels.

METHODS

Tissue Culture

Isolation, dissociation and culture of BFSG neurons was done under aseptic conditions as described previously (Lei *et al.*, 1997; Lei *et al.*, 1998). Neurons were dissociated by incubation with trypsin (Sigma) and type 1A collagenase (Sigma) for 42-45min, at 37°C. Final dissociation was accomplished by tituration with a 1 ml Pasteur pipette. Cells were suspended in 3 ml of serum-free, modified L-15 medium (73% L-15 (Gibco), 10 mM glucose, 1 mM $CaCl_2$, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μM cytosine arabinoside; pH 7.2). The dissociated cells were then redistributed into 2.5 ml of medium in each of 20 35-mm polystyrene tissue culture dishes (Nunc). Dishes were placed in a light proof, humidified chamber and maintained at room temperature (22°C) for 6-7days, unless otherwise stated.

In Vivo Experiments

The tonic influence of LHRH *in vivo* was prevented by cutting preganglionic C-fibers which are the sole source of LHRH in BFSG (Jan *et al.*, 1979; Jan *et al.*, 1980b; Jones, 1987; Jobling & Horn, 1996; Thorne & Horn, 1997; Troskie *et al.*, 1997). Bullfrogs were anesthetized by injecting a 0.01 % solution of tricane methane sulfonate (MS-222) into the dorsal sac. Under aseptic conditions, a rostral-caudal incision was made through the ventral skin and body wall. Internal organs were held aside and the

rami communicates leading from both the VIIth and VIIIth spinal nerves were cut on one side of the animal (ipsilateral). This selectively destroys preganglionic C-fibers which contain LHRH without affecting B-fibers. Thus, normal cholinergic transmission from preganglionic B-fibers to B-cells is maintained. A 1-2 mm section of nerve was removed to prevent re-innervation. The C-fibers on the contralateral side were not cut and this side served as a control for the cut side. The ventral cut through the body wall and skin was sutured closed and the animal was allowed to recover. 10 d post surgery the bullfrog was killed by pithing and the VIIIth-Xth ganglia were removed separately from each side. B-neurons from the cut C-fiber side and contralateral sides were enzymatically dissociated and plated in two sets of polystyrene tissue culture dishes (see above) for acute electrophysiological investigation.

Electrophysiology

Discontinuous, single-electrode, voltage clamp methods (Axoclamp 2A; Axon Instruments) were used to record whole-cell Ca^{2+} channel currents. As described previously, Ba^{2+} was used as the charge carrier to monitor Ca^{2+} channel activity (I_{Ba}) (Jassar *et al.*, 1993; Lei *et al.*, 1997). Data were acquired with a TL-1 DMA interface acquisition system operating under software control (pClamp 5.0; Axon Instruments), and filtered to –3 db at 3 kHz. For recordings, media was exchanged with 'external' solution containing (in mM) 117.5 N-methyl-D-glucamine (NMG) chloride, 2.5 NMG-HEPES, 2.0 $BaCl_2$ (pH 7.2). The flow rate was set so that complete exchange of media for external Ba^{2+} solution was accomplished in 1-2 min. Low resistance (~4 MΩ D.C. resistance, as measured in 'external' solution) fire-polished electrodes were used and filled with 'internal' solution consisting of (in mM) 76.5 NMG-chloride, 2.5 HEPES, 10 Tris(hydroxymethyl) aminomethane (Tris)-bis-(o-aminophenoxy)-N,N,N',N'-tetracetic acid (BAPTA), 5 Tris-ATP, 4 $MgCl_2$ (pH 7.2). In cases where the acute effects of LHRH on I_{Ba} was examined, 0.4mM GTP was added to the 'internal' solution.

Whole-cell recordings were first made in bridge-balance, current-clamp mode, prior to switching to single-electrode, discontinuous voltage-clamp mode for the study of I_{Ba}. The holding potential (V_h) was –90 mV, unless otherwise stated. Input capacitance was used as a measure of cell size. Cells with input capacitance (C_{in}) >30 pF were

selected for recordings. As C_{in} of BFSG C-cells is <20 pF (Kurenny *et al.*, 1994), it is assumed that all recordings were made from B-cells. C_{in} was calculated by integrating the capacitive transient induced by a 10mV depolarizing step from –90 mV. Peak current measurements were subtracted using a P/4 protocol (Jones & Marks, 1989a). Illustrated traces were filtered digitally at 1 KHz post-hoc and presented using Microcal Origin 6.1 software.

Electrophysiological recordings were made from acutely-dissociated BFSG B-neurons (acute), or neurons cultured for 6-7 d in either the absence (6 d culture) or presence of 0.45 μM LHRH (6d LHRH). This culture time was selected as the effect of LHRH on I_{Ba} was significant at that time (see Figure 4-2). To study the signal transduction pathway associated with the LHRH receptor, recordings were made from control neurons cultured for 6d in the presence of various inhibitors alone or 6d LHRH-treated cultured neurons in the presence of the inhibitors. In experiments where the acute effects of LHRH or noradrenaline (NA) on I_{Ba} were examined, the peptide was bath applied via a perfusion system (2 ml/min), which allowed for complete exchange of solutions within 1-2 min (as measured by maximal I_{Ba} suppression). Unless otherwise stated, all values reported are peak current density (peak I_{Ba}, recorded at –10 mV, normalized to cell size; C_{in}). Current density takes into account the variability of cell size, as the size of the neuron is proportional to the size of its current.

Chemicals

LHRH (chicken-II GnRH) was purchased from Peninsula Labs (Belmont, CA). Leibovitz's L-15 medium and penicillin-streptomycin antibiotics were from GIBCO BRL. The following were from Biomol (Plymouth Meeting, PA): genistein, diadzein, wortmannin, PP1, PD-98059 (2'-Amino-3'-methoxyflavone), perillic acid (PA), α-Hydroxyfarnesylphosphonic acid (α-HFA), H-89 (N-[2-(p-Bromocinnamylamina)ethyl]-5-isoquinolinesulfonamide-2HCl), Chelerythrine (chelerythrine chloride), U73122, U73343, Sp- and Rp- cAMPS (-Adenosine-3',5'-cyclic mononphosphorothioate triethylamine salt), PMA (Phorbol 12-myristate-13-acetate) and 4α-phorbol (4α-phorbol 12-myristate-13-acetate) and actinomycin-D. ω-Conotoxin-GVIA was from Alomone Labs (Jerusalem, Israel). Nifedipine was obtained from Tocris (Ballwin, MO). All other

chemicals were from Sigma (St. Louis, MO). Some inhibitors, being highly hydrophobic, were dissolved in DMSO to make stock solutions. Stock solutions were dissolved in culture media to make final desired concentrations. The final DMSO concentration used in all cases was ≤ 0.1 %. This concentration of DMSO did not affect I_{Ba} as Ca^{2+} currents recorded from 6 d cultured cells in the presence or absence of DMSO were not significantly different from controls (data not shown, n=10). Inhibitors were used at 5-10 times their published K_D or IC_{50} values for their cognate enzymes. LHRH and/or enzyme inhibitors when used, were added to media at the time of dissociation, and cultured in their presence for 6-7 d. Cells treated with inhibitor plus LHRH (0.45 μM) were pretreated for 45 min with the inhibitor before addition of LHRH. Culture media were changed daily to maintain effective concentrations of LHRH and inhibitors. Media containing wortmannin and PD98059 were changed every 8 hours, in order to maintain effective concentrations, since both of these chemicals are subject to hydrolysis in aqueous solutions (Kimura *et al.*, 1994; Virdee & Tolkovsky, 1996). In order to maintain activation and not down-regulation of PKC (Matthies *et al.*, 1987), PMA (and the negative control 4α-phorbol) were used at 80 nM and applied to neurons for 1 hr/day, followed by several washes to remove any residual drug.

All data are presented as means±SEM and were considered significant when $p<0.05$ using Student's unpaired, two-tailed, t-test or one-way analysis of variance with Student-Newman-Keuls test for post hoc comparison.

RESULTS

Neurotrophic Effects of LHRH on Ca^{2+} channels

Figure 4-1 illustrates typical recordings of I_{Ba} from an acutely-dissociated BFSG B-cell, a cell maintained in culture for 6 days, and a cell maintained in culture for 6 days in the presence of LHRH (0.45 μM). Cells were held at –90 mV and currents were evoked by a series of incremental 10 mV depolarizing steps; tail currents were recorded at –40 mV to slow their kinetics and facilitate their analysis. 6 d of culture itself caused an increase in I_{Ba} as compared to that recorded from acutely dissociated cells. A further, substantial increase in I_{Ba} amplitude was seen in cells that had been cultured for 6 d with 0.45 μM LHRH. These effects are illustrated in Figure 4-1 are further quantified in

Figure 4-2. Acutely dissociated cells exhibited 3.9±0.3nA total peak I_{Ba} at –10 mV (n=31) (Fig 4-2A) whereas after they were maintained in serum-free defined medium culture for 6d, the current increased to 9.1±0.4 nA (n=49) ($p<0.05$) (Fig 4-2A). This finding confirms the previous observation that a transient increase in peak current occurs, during the first 3-6 days of culture (Lei *et al.*, 1997). After BFSG B-cells have been maintained in culture for 15 days, the total I_{Ba} current seen is similar to that of acutely dissociated cells (Lei *et al.*, 1997). Inclusion of 0.45 μM LHRH in the culture media significantly increased the total peak I_{Ba} of 6d cultured cells (11.7±0.7 nA; n=35) over that seen in cells maintained in culture in the absence of LHRH ($p<0.05$) (Fig 3-2A).

As previously noted (Lei *et al.*, 1997), B-cells maintained in culture for >2 days begun to sprout processes and this coincided with an increase in input capacitance (C_{in}). After 6d in culture, there was a greater than two-fold increase in C_{in} ($p<0.05$) (Fig. 4-2B). Inclusion of 0.45 μM LHRH did not appear to affect the growth and production of neurites or soma as no significant difference in cell capacitance was measured ($p>0.05$) (Fig. 4-2B).

The effect of LHRH on peak current density (peak I_{Ba} normalized to cell size; C_{in}) was examined. Current density takes into account the variability of cell size; as the size of the neuron is proportional to the size of its current. Culturing B-cells for 6d had no effect on I_{Ba} peak current density ($p>0.05$) (Fig. 4-2C). Thus, the increase in peak I_{Ba} (Fig 4-2A) was balanced by the increase in cell size so that there was no appreciable change in density. However, inclusion of 0.45μM LHRH in the culture media increased the peak current density (175.4±16.4pA/pF; n=35) by 33 % compared to cells cultured in the absence of LHRH (117.7±6.8pA/pF; n= 49; $p<0.05$) and by 42 % compared to acutely dissociated cells (100.6±7.3 pA/pF; n=31; $p<0.05$) (Fig. 4-2C).

LHRH Does Not Alter Voltage-Dependence of Ca^{2+} Current Activation or Inactivation

Figures 4-3A-C illustrate the I_{Ba} density-voltage relationships for acutely dissociated cells, and cells cultured for 6 days in the presence or absence of 0.45μM LHRH. Neurons were held at V_h of –90 mV, -60 mV and –40 mV. The peak current for all three groups occurred at –10 mV, implying that the manipulations had little if any effect on the activation kinetics (the voltage-dependence of Ba^{2+} conductance (g_{Ba})

activation). This was confirmed by analyzing the voltage-dependence of activation obtained from tail current amplitudes (Fig 4-4A). Tail current amplitudes were estimated by fitting tail currents during the 2-7 mseconds after the step to -40 mV to a monoexponential function and extrapolating the amplitude at zero time. Using this protocol it was possible to extrapolate the peak tail current in the absence of contaminating capacitative currents. Tau values for this curve are a good measure of the 'quality' of the tail current, as there should be no variation between cells under ideal clamp conditions. As there was some variation in the τ values recorded from both 6 d culture and 6 d LHRH treated cells, the recorded tail currents and the derived voltage-dependence of activation from these cells must be interpreted with caution. No differences occurred between the normalized activation curves for acutely dissociated neurons, control neurons cultured for 6 d, or LHRH-treated 6 d cultured neurons (Fig 4-4A). All test conditions had curves which well matched the activation curve fitted with Boltzman kinetics (Fig 4-4A). Changing the V_h from –90 mV to –40 mV produced a similar reduction in the peak I_{Ba} density for acutly dissociated cells, as well as cells cultured for 6 d either in the presence or absence of LHRH (Figs 4-3A-C), suggesting that there was no alteration in the steady-state inactivation of I_{Ba}. This is also reflected in the isochronic-inactivation curves (Fig 4-4B). For 4 sec prior to the test pulse to –10 mV, cells were held at a range of prepulse potentials from –105 to –30mV. The test pulse current (normalized as I/I_{max}) is plotted against the holding potential (V_h) of the prepulse. This does not yield a true 'h_∞' plot as the removal of inactivation of g_{Ca} is very slow (Jones & Marks, 1989b). There was no difference between the normalized inactivation curves for 6d cultured cells in the presence or absence of LHRH (Fig. 4-4B). Compared with acutely dissociated cells, the isochronic-inactivation plots for 6d cultured cells both with and without LHRH were shifted to the left (Fig 4-4B). The effect of LHRH on g_{Ba} inactivation kinetics thus differs from that of NGF, as NGF induced a rightward shift in the curve so as to restore the kinetics of the Ca^{2+} channels to those of acutely isolated neurons (Lei *et al.*, 1997).

Long-term exposure to LHRH therefore caused an increase in the total amount of I_{Ba}/g_{Ba} without altering either activation or inactivation, and without affecting cell size.

Increases in conductance, in the absence of changes in inactivation, would be consistent with an increase in the total number of functional Ca^{2+} channels available.

Functional LHRH Receptors Present at Cell Surface After 6d Culture with LHRH

Prolonged exposure of BFSG B-neurons to LHRH throughout the 6 d time course of our culture experiments may promote desensitization (Bosma & Hille, 1989), internalization and/or alterations in expression of functional LHRH receptors or the cell membrane. We therefore used the acute, inhibitory effects of LHRH on I_{Ba} (Elmslie *et al.*, 1990) to assess the level of functional LHRH receptors, during the course of our experiments. Cells were examined 18-24 h after the last daily replenishment of medium containing 450 nM LHRH.

Cells maintained in the prior presence of 450 nM LHRH for 6days showed significantly reduced I_{Ba} suppression by acutely applied 1 μM LHRH (2.83 nA), a supression of 36.2% as compared to control cultured cells where 48.2% suppression (3.16 nA) was seen ($p<0.05$) (Fig 4-5A). In both cases, there was a kinetic slowing of the current and a reduction in the level of inactivation seen (data not shown) which supports the hypothesis that LHRH acts through a G-protein coupled receptor to acutely inhibit Ca^{2+} currents (Diverse-Pierluissi *et al.*, 1995). This implies that functional receptors persist on BFSG neurons throughout the 6-7day period of chronic peptide exposure. The reduced responsiveness may imply some desensitization, down-regulation or internalization of LHRH receptors *per se* or some general down regulation of G-protein coupled receptor signaling. To distinguish whether the reduction in LHRH-induced I_{Ba} suppression in 6 d LHRH cultured cells was due to LHRH receptor desensitization or due to an alteration in receptor/G-protein/Ca^{2+} channel signaling, a different GPCR agonist which also induces suppression of Ca^{2+} channels was tested. Noradrenaline (NA; 100 μM) induced suppression of I_{Ba} to a similar extent in 6 d cultured (33.4±1.6 %; n=6) and 6d LHRH cultured neurons (31.9±2.2 %; n=7; $p>0.05$) (Fig 4-5B). This suggests that there is not an alteration is G-protein-Ca^{2+} channel signaling in LHRH cultured neurons, and that most likely the reduction in LHRH-induced I_{Ba} suppression seen in LHRH cultured neurons was due to receptor desensitization. However, in spite of

desensitization, acutely applied LHRH is able to suppress I_{Ba} in neurons chronically treated with LHRH for 6-7 days, suggesting the presence of functional LHRH receptors.

Regulation of N- and L- Type Ca^{2+} Channels by LHRH

In BFSG, N-type channels carry ~90% of the total I_{Ca}, while L-type channels carry most of the remaining amount (Jones & Elmslie, 1992). Studies in pheochromocytoma (PC12) cells and in BFSG neurons have shown that the nifedipine-sensitive L-type Ca^{2+}current ($I_{Ca,\ L}$) and the ω-conotoxin GVIA-sensitive N-type Ca^{2+} current ($I_{Ca,\ N}$) are differentially regulated by NGF (Lei *et al.*, 1997; Lewis *et al.*, 1993). We therefore compared the pharmacology of I_{Ba} recorded from 6 d cultured and 6 d LHRH-treated cells, to see if LHRH was also capable of differentially regulating these two types of Ca^{2+} channels in BFSG. Currents were generated once every 60 sec by voltage steps to 0mV (from V_h -90 mV). Cells cultured for 6-7 d with or without LHRH were first exposed to ω-Conotoxin-GVIA (ω-CgTX; 300 nM) (for 45 min to allow for full inhibition of N-type Ca^{2+} channels to develop) followed by 15 min of 1 μM nifedipine. The contribution of L-, N- type Ca^{2+} channels to total I_{Ba} was determined as the amount of total current density suppressed by ω-CgTX or nifedipine in individual cells. The current density that remained after ω-CgTX and nifedipine was defined as $I_{Ba,\ other}$. In control cultured cells (n=9) the ω-CgTX-sensitive current density was 45±8.8 pA/pF (67.4±6.9 %), 11.3±3.8 pA/pF (20±5.5 %), 20±5.5 % was nifedipine-sensitive and 7.5±1.3 pA/pF (12.6±3.6 %) was ω-CgTX and nifedipine resistant (Figure 4-6). In LHRH treated cells (n=10) 67.5±6.5 pA/pF (85±3.5 %) of the current was ω-CgTX-sensitive, 5.0±1.3 pA/pF (6.7±2.6 %) was nifedipine-sensitive, and 6.3±1.3 pA/pF (8.2±1.6 %) was ω-CgTX- and nifedipine-resistant (Figure 4-6). LHRH thus induced a significant increase in $I_{Ba,\ N}$ ($p<0.05$), whereas $I_{Ba,\ L}$ ($p>0.1$) and $I_{Ba,\ other}$ ($p>0.4$) were unchanged. Although NGF also increases $I_{Ba,\ N}$ in BFSG neurons, its effect differs from that of LHRH as NGF also produces a large increase in $I_{Ba,\ L}$ (Lei *et al.*, 1997).

In vivo Cut C-Fibers

We next examined whether the effects of LHRH that we observed *in vitro* could also be seen *in vivo*. As mentioned above, preganglionic B-fibers emerge from the CNS

in the IVth, Vth, and VIth spinal nerves, whereas preganglionic C-fibers emerge in the VIIth and VIIIth spinal nerves (Horn & Stofer, 1988) . By selectively cutting the rami communicantes connecting the VIIth and VIIIth spinal nerves with their associated ganglia it was possible to selectively interrupt C-fiber transmission whilst leaving B-cell/B-fiber cholinergic transmission intact (Fig 4-7A and B). This manipulation prevents the tonic release of LHRH from C-fiber terminals, as it is known that five days after denervating preganglionic C-fibers, 95 % of ganglionic LHRH is removed (Jan *et al.*, 1979). Acutely dissociated B-neurons from BFSG that had preganglionic C-fibers cut for 10d exhibited peak I_{Ba} density of 127.4±10.3 pA/pF (n=20) (Fig 4-7E). Recordings from a representative cell are illustrated in Figure 4-7D. This was significantly less than the current density seen in B-cells derived from the contralateral control side of the same animals (176.2±22.3 pA/pF; n=18; $p<0.05$) (Figure 4-7E). Thus the absence of LHRH caused a 38 % decrease in the availability of I_{Ba}. Recording from a representative cell are illustrated in Figure 4-7C. In spite of changes seen in current density, cutting C-fibers had no effect on either the activation or inactivation kinetics (data not shown).

Transduction Mechanisms for Neurotrophic Regulation of I_{Ba} by LHRH

Next examined were the signaling pathways through which the LHRH receptor promotes an increase in the functional expression of Ca^{2+} channels.

LHRH-Induced Changes are Transcription Dependent

A DNA-transcription inhibitor (actinomycin-D, 0.02 μg/ml) was used to test whether LHRH-induced changes in I_{Ba} involved an alteration in gene expression. The effect of this concentration of actinomycin-D on the initial increase in I_{Ba} amplitude and cell size (C_{in}) was not significant. Thus, I_{Ba} in acutely-dissociated cells was 3.9±0.3 nA (n=35) and this increased to 9.1±0.4 nA (n=49) after 6d in culture (Fig 4-1A and B) and to only 7.6±0.4 nA in the presence of actinomycin-D (n=14, $p>0.05$ compared to 6d cultures). C_{in} of acutely dissociated cells was 41.2±3.0 pF (n=35). This increased to 85.1±3.6 pF (n=49) in 6 d cultures (Fig 4-2B) but to only 72.8±4.8 pF in cells cultured for 6d with actinomycin-D (N=14, $p>0.05$ compared to control 6d cultures). I_{Ba} density was unchanged. Thus I_{Ba} density after 6 d in actinomycin-D was 104.9±7 nA/pF (n=17;

Fig 4-8) compared to 117.7±6.8 nA/pF (n=49) in 6d cultured cells ($p>0.5$). Actinomycin-D did however prevent the LHRH-induced increase in current density. Accordingly, the current density of BFSG B-neurons recorded in the presence of actinomycin-D for 6d was indistinguishable from that of neurons recorded in the presence of actinomycin-D plus LHRH for 6d (105.2±6.8 pA/pF, n=26, $p>0.15$, Fig 4-8). This suggests that LHRH induces alterations in the level of functional Ca^{2+} channels via signaling through the nucleus to alter gene expression.

Inhibition of the Effect of LHRH on Ca^{2+} Channels by Inhibition of Ras

Effective inhibition of Ras function can be obtained with use of the isoprenylation blockers α-hydroxyfarnesylphosphonic acid (α-HFA) (IC_{50} = 30 nM in isolated enzyme assays) (Gibbs *et al.*, 1993; Kohl *et al.*, 1993) and the limonene metabolite, perillic acid (PA) (IC_{50} = 6.6 μM in isolated enzyme assays) (Crowell *et al.*, 1991). Both substances impair the effects of NGF on the functional expression of I_{Ca} in BFSG neurons (Lei *et al.*, 1998) Addition of α-HFA (10 μM) to the culture media prevented the LHRH-induced increases in I_{Ba} density, so that current densities were not significantly different from α-HFA treated control (Table 4-1; Fig 4-9) ($p>0.05$). α-HFA had no affect on control I_{Ba} density (Table 4-1). Although PA (100 μM) significantly reduced control I_{Ba} density ($p<0.05$) (Table 4-1; Fig 4-9), the inclusion of LHRH while in the presence of PA, did not produce an increase in current density above that seen in the presence of the inhibitor alone ($p>0.05$) (Table 4-1; Fig 4-9). The results with PA and α-HFA are thus consistent with the involvement of Ras in the potentiation of Ca^{2+} currents by LHRH.

Involvement of MEK in the LHRH Response

Once activated, Ras initiates a kinase cascade which eventually proceeds to the phosphorylation of the MAP kinase (ERK). ERKs are activated via dual phosphorylation by the tyrosine/threonine kinase family of MEKs (for review see (Seger & Krebs, 1995). The involvement of MEK in LHRH-mediated changes in Ca^{2+} channel expression was examined. MEK is selectively inhibited both *in vivo* and *in vitro* by PD98095 (IC_{50} = 2 μM in cell culture assays) (Alessi *et al.*, 1995; Dudley *et al.*, 1995). Cells were treated with 10 μM PD98059, either alone, or in combination with LHRH. I_{Ba} density recorded

in the presence of PD98059 was not significantly different from that of control (P>0.05) (Table 4-1; Fig 4-9). However, PD98059 blocked the LHRH-induced increase in current densities (p<0.05) (Table 4-1; Fig 4-9), which is consistent with the activation of MEK, and thus presumably the activation of ERK, in the increase in functional Ca^{2+} channels by LHRH.

PKC and PLC are Required to Mediate the Effects of LHRH

In pituitary cell lines, GnRH receptor signaling to activate ERK has been shown to involve PLC, and PKC (Sim *et al.*, 1995; Reiss *et al.*, 1997 and see reviews Stojilkovic & Catt, 1995; Shacham *et al.*, 1999; Naor *et al.*, 2000). We therefore examined the role of PKC in LHRH-induced potentiation of I_{Ba} in BFSG B-cells, with the use of the PKC inhibitor chelerythrine (IC_{50} = 0.6 μM in isolated enzyme assays) (Herbert *et al.*, 1990). At 1 μM, chelerythrine reduced I_{Ba} density as compared to control cells (P<0.05) (Table 4-1; Fig 4-10). However at this concentration, the inhibitor was also able to effectively prevent LHRH-mediated increases in current density, as I_{Ba} recorded also in the presence of chelerythrine plus LHRH were not significantly different from cells cultured with chelerythrine alone (P>0.05) (Table 4-1; Fig 4-10). These results are consistent with the hypothesis that LHRH signaling involves PKC.

The PLC inhibitor U73122 (IC_{50} = 2 μM in cell culture assays), together with its inactive structural analog U73343, provides a specific way to investigate the role of PLC in signal transduction. U73122 inhibits agonist-induced PLC activation in BFSG neurons (Stemkowski *et al.*, 2002) and inclusion of 0.45 μM LHRH failed to increase I_{Ba} density, while cells were in the presence of 20 μM U73122 (67.3±5.3 pA/pF (n=26) compared to 63.4±4.7 pA/pF (n=25) in the presence of U73122 alone; p>0.05, table 4-1; Fig 4-10). However a substantial increase in Ca^{2+} current density occurred when cells maintined for 6d in the presence of 0.45 μM LHRH (120.1±16.4 pA/pF (n=18) and the inactive analogue U73343 (20 μM) (compared to 67.2±7.3 pA/pF (n=23) (p<0.05) in the presence of U73343 alone; table 4-1; Fig 4-10).

Role of PKA in Mediating the Effects of LHRH

In some pituitary cell lines, the PKA signal transduction pathway is involved in mediating the actions of GnRH (Kuphal *et al.*, 1994; Han & Conn, 1999). Furthermore, signaling pathways involving PKA, and/or cAMP are known to affect the Ras/ERK cascade (see Impey *et al.*, 1999; Naor *et al.*, 2000 for review). These findings prompted us to determine whether LHRH-induced alterations in Ca^{2+} channel expression involved PKA.

To evaluate the role of PKA in LHRH-mediated Ca^{2+} channel induction, cells were treated with 1 μM H-89 alone or with LHRH. H-89 is a known selective inhibitor of PKA (K_i = 48 nM in isolated enzyme assays) (Chijiwa *et al.*, 1990). H-89 alone had no affect on I_{Ba} density (Table 4-1; Fig 4-10). Cells treated with H-89 plus LHRH did not show the increase in current density that was seen with LHRH alone ($P<0.05$) (Table 4-1; Fig 4-10), which supports the idea that PKA is also involved in the LHRH response.

Inhibitors of Src Kinase and PI3Kinase Do Not Prevent LHRH-Mediated Changes in I_{Ba}

It has recently been found that certain G_i coupled GPCRs, via $G_{\beta\gamma}$ subunits, can cause the activation of ERK via the transactivation of growth factor receptors by a process that is believed to involve the activation of the PI3K-γ and the non-receptor tyrosine kinase c-Src (Koch *et al.*, 1994; van Biesen *et al.*, 1995; Lopez-Ilasaca *et al.*, 1997; Takeda *et al.*, 1999; Eguchi *et al.*, 1998). The role of Src kinase in LHRH-mediated Ca^{2+} channel regulation was investigated with the use of PP1, an inhibitor of the Src kinases $p56^{lck}$ (K_i = 6 nM) and $p59^{fyn}$ (K_i = 170 nM) (Hanke *et al.*, 1996). Inhibition of Src by 1.5 μM PP1 failed to prevent LHRH from potentiating I_{Ba} density, as there was a significant increase ($P<0.05$) in current density with PP1 plus LHRH (94.0±10.1 pA/pF) compared to the current density seen in PP1 alone (44.1±2.5 pA/pF) (Table 4-1; Fig 4-12). The role of PI3K in LHRH-mediated Ca^{2+} channel regulation was also examined with the PI3K inhibitor wortmannin (IC_{50} = 50 nM in cell culture assays) (Yano *et al.*, 1993), which we have previously shown in BFSG to be an effective inhibitor of PI3K-mediated Na^+ channel regulation by NGF (Lu et al., 2002). A similar result was seen with the use of wortmannin as was seen with PP1, in that 100 nM wortmannin failed to prevent a significant increase in I_{Ba} density when BFSG B-cells

were stimulated with LHRH (72.1±8.4 pA/pF; 48.3±6.2 pA/pF for wortmannin alone) (Table 4-1) ($P<0.05$; Fig 4-11). This suggests that PI3K-γ and c-Src are not involved in the LHRH-mediated increases in Ca^{2+} channel expression, as inhibition of these pathways failed to prevent a LHRH-mediated potentiation of current density.

Direct activation of PKC, but not PKA potentiates I_{Ba} density

The results with inhibitors suggest that the LHRH-R signals through both PKC and PKA to cause activation of ERK which in turn leads to an increase in I_{Ba} density (see above). In order to confirm this, PKC and PKA were directly stimulated in an attempt to mimic the action of LHRH. Previous studies have shown that direct PKC activation by phorbol esters can induce ERK activation (Thomas *et al.*, 1992; Hawes *et al.*, 1995;). B-Cells treated with the phorbol ester PMA (80 nM) to activate PKC (Ryves *et al.*, 1991) exhibited current densities (70.8±5.8 pA/pF; n=17) that were significantly greater than neurons treated with 4α-phorbol, the negative control for PMA (55.4±4.3pA/pF; n=17) (Fig 4-12 and 4-13). Thus, direct activation of PKC is sufficient to cause an increase in the level of Ca^{2+} channels expressed in BFSG. Unlike PKC, the direct activation of PKA was not sufficient to increase I_{Ba} density. Current densities recorded from cells treated with the cAMP analogue Sp-cAMPS (10 μM) (57.3±4.2 pA/pF; n=41) were no different than the current density recorded from cells treated with the enantiomer Rp-cAMPS (10 μM), a competitive inhibitor of the activation of PKA by cAMP, (63.8±2.8 pA/pF; n=42; $p>0.05$) (Fig 4-13). This, taken with the results from the inhibition of PKA and PKC during stimulation with LHRH suggests that in the regulation of Ca^{2+} channels by LHRH both pathways are necessary, yet only PKC on its own is sufficient to produce these effects.

DISCUSSION

The primary finding of this chapter is that long-term application of the neuropeptide transmitter LHRH upregulates Ca^{2+} channel density in fully differentiated, adult sympathetic neurons both *in vitro* and *in vivo*. Pharmacological experiments are consistent with the involvement of the Ras-ERK pathway in this effect. Removing all ganglionic sources of LHRH *in vivo*, by surgically cutting pre-ganglionic C-fibers

reduces I_{Ba} density. These findings suggest that, in addition to its previously known role as a ganglionic neurotransmitter, LHRH also functions in a neurotrophic manner to regulate the functional expression of Ca^{2+} channels in BFSG.

Regulation of I_{Ba} by LHRH

The predominant effect of LHRH was an increase in $I_{Ba,\ N}$ density (Fig 4-6). Since $I_{Ba,\ N}$ is the dominant contributor to total I_{Ba} in BFSG (Jones & Elmslie, 1992), an increase in total current densitiy was observed (Fig 4-2C).

Since LHRH had no effect on activation or inactivation kinetics, the peptide is unlikely to have exerted its effects on the expression of Ca^{2+} channel β subunits or other proteins that may effect channel properties (Varadi *et al.*, 1991). It is tempting to speculate therefore that LHRH increased the functional expression of N-type Ca^{2+} channel α-subunits. It is of course possible that the peptide promoted expression of channels with greater single channel conductance, this possibility remains to be tested by recording single channel activity.

Signal Transduction Mechanism for Effect of LHRH

The LHRH-R, depending on the cell used, has been reported to activate different G proteins/signal transduction pathways (Conn *et al.*, 1979; Kuphal *et al.*, 1994). The mammalian GnRH receptor, in mammalian cells and cell lines, couples to $G_{q/11}$, G_s and G_i (Hsieh & Martin, 1992; Hawes *et al.*, 1993; Janovick & Conn, 1994; Stanislaus *et al.*, 1997; Stanislaus *et al.*, 1998; Ulloa-Aguirre *et al.*, 1998). In BFSG, LHRH increases IP_3 turnover and intracellular Ca^{2+} concentrations (Pfaffinger *et al.*, 1988), suggesting that the LHRH receptor is coupled to $G_{q/11}$ proteins. A novel mammalian receptor has been discovered for the type II GnRH (chicken II-GnRH) (Millar *et al.*, 2001) which may correspond with the BFSG receptor. However it is not known whether this is the only receptor used by LHRH for signaling or if $G\alpha_{q/11}$ is the primary and/or only G-protein used by the BFSG receptor. As the ERK cascade can be activated by many different components of the GPCR signaling cascade (see reviews by (Gutkind, 1998; Naor *et al.*, 2000), muliple signaling pathways may link the LHRH-R to the ERK cascade.

The downstream targets for LHRH regulation of Ca^{2+} channels likely include the Ras-MEK-ERK pathway. This suggestion is based on positive results with α-HFA, perrilic acid, and PD98059. The GnRH-R is known to cause activation of ERK1/2 via the PKC pathway (Sim *et al.*, 1995; Sundaresan *et al.*, 1996; Reiss *et al.*, 1997). Direct stimulation of PKC with the phorbol ester is known to directly activate ERK (Reiss *et al.*, 1997). The results with U73122 and chelerythrine suggest that in BFSG B-neurons the LHRH-R signals via PLC and PKC (Table 4-1) furthermore, direct simulation of PKC is sufficient to cause an increase in I_{Ba} (Fig. 4-13) suggesting that PKC is both necessary and sufficient to regulate BFSG Ca^{2+} channels. The result with PMA is contrary to the effect previously reported that chronic activation of PKC by phorbol esters reduces Ca^{2+} channel expression in sympathetic neurons (Przywara *et al.*, 1997). However in that report phorbol esters were chronically applied, which is known to downregulate PKC (Matthies *et al.*, 1987), as opposed to our protocol which would favor the activation of PKC and not its down regulation. In both neurons and PC12 cells, cAMP activates ERK1/2 in a Ras-independent manner via the activation of a Rap1 and B-Raf dependent pathway by PKA (Vossler *et al.*, 1997; Dugan *et al.*, 1999; Ambrosini *et al.*, 2000; Grewal *et al.*, 2000). LHRH regulation of I_{Ba} was dependent on PKA (Table 4-1), which suggests that in B-neurons LHRH signals via the above pathway to activate ERK1/2 via the Rap1/B-raf pathway. As the direct application of the cAMP analog Sp-cAMPS (Fig 4-13) failed to reproduce the effects of LHRH, yet the PKA inhibitor H-89 prevented the LHRH effect, it can be concluded that the cAMP system is necessary for LHRH signaling to regulate I_{Ba} yet not sufficient on its own to mimic these effects.

Certain G_i-coupled GPCRs can cause activation of ERK via the trans-activation of growth factor receptors by a process that is believed to involve the activation of PI3K-γ and the non-receptor tyrosine kinase c-Src (Koch *et al.*, 1994; van Biesen *et al.*, 1995; van Biesen *et al.*, 1996; Lopez-Ilasaca *et al.*, 1997; Eguchi *et al.*, 1998; Takeda *et al.*, 1999). Because the effect of LHRH was not inhibited by inhibition of either PI3K or c-Src, the possibility that LHRH signaled through these pathways is unlikely. However with the use of the c-Src inhibitor PP1 and the PI3K inhibitor, wortmannin, current densities recorded in the presence of the inhibitor alone were significantly smaller than control currents (Table 4-1), suggesting that PI3K-γ and c-Src may be involved in some

aspect of basal Ca^{2+} channel regulation in BFSG B-cells. Alternatively, the effects of PI3K and c-Src inhibition may reflect a reduction in 'health' of the neurons. In sympathetic neurons, the PI3K pathway is important for activating survival pathways initiated by certain growth factors (Crowder & Freeman, 1998; Mazzoni *et al.*, 1999; Kelly-Spratt *et al.*, 2002) and the inhibition of PI3K blocks growth factor-induced survival. BFSG neurons, while not dependent upon exogenous neurotrophins, may rely upon endogenously active PI3K for some aspects of health and/or survival. Thus, the effects of PP1 and wortmannin may not be directly due to alterations in Ca^{2+} channel regulation, but may reflect the 'unhealthy' state of the cell. The result with actinomycin D (Table 4-1) show that gene transcription is required for the LHRH-mediated regulation of Ca^{2+} channels, and suggest that ERK may interact with the nucleus to cause an increase in the synthesis of new Ca^{2+} channel proteins, most likely ω-CgTX-sensitive N-type Ca^{2+} channels.

Figure 4-14 summarizes the possible signaling pathways utilized by the LHRH-R in mediating the increase in Ca^{2+} channel synthesis.

Comparison of Actions of NGF and LHRH

NGF increases peak I_{Ba} density in cultured adult BFSG B-neurons (Lei *et al.*, 1997). This involves an increase in both N- and L-type Ca^{2+} channel I_{Ba} ($I_{Ba,\,N}$, $I_{Ba,\,L}$), and a decrease in inactivation of the total Ba^{2+} conductance (Lei *et al.*, 1997). The effect of LHRH on g_{Ba} is different than the effect of NGF. While there is an increase in the total g_{Ba} and an increase in $I_{Ba,\,N}$ with LHRH, there is no decrease in inactivation of the total Ba^{2+} conductance. Furthermore, NGF appears to promote an increase in $I_{Ba,\,L}$ (Lei *et al.*, 1997) while LHRH did not alter $I_{Ba,\,L}$. Like the effect of NGF on both PC12 cells and adult BFSG B-cells, LHRH may also signal via the Ras pathway to regulate Ca^{2+} channels (Pollock & Rane, 1996; Lei *et al.*, 1998;). However, in PC12 cells, sustained activation of $p21^{ras}$ alone was not sufficient on its own to increase Ca^{2+} current (Pollock & Rane, 1996), suggesting that multiple signaling pathways in addition to Ras are required for Ca^{2+} channel regulation.

Although the actions of LHRH are reminiscent of those of NGF (Lei *et al.*, 1997), its action in up regulating Ca^{2+} channels does not seem to be merely a consequence of

improved 'health' of cultured B-cells. Cells cultured in either the presence or absence of LHRH showed no visible differences, nor in the extent or dimensions of axonal sprouting (data not shown). This effect is similar to that of the action of other neurotrophins in modulating ion channels with out the initiation of a growth response in basal forebrain neurons (Levine *et al.*, 1995) and in BFSG (Lei *et al.*, 1997; Lei *et al.*, 2001). Furthermore, the effects of LHRH in up regulating I_{Ba} density appear to be specific for only certain types of ionic currents in BFSG, since there is no effect of culturing BFSG B-neurons in the presence of LHRH on Na^+ channel currents (Lu et al, 2002).

Physiological Significance

Previous reports have shown that activation of ERK is important for the regulation of various ion channels (Huang & Rane, 1994; Pollock & Rane, 1996; Fitzgerald & Dolphin, 1997; Strobeck *et al.*, 1999). Various growth factors also regulate ion channels in adult sympathetic neurons (Lei *et al.*, 1997), action potential duration in mature sensory neurons (Chalazonitis *et al.*, 1987), as well as Na^+, K^+, and Ca^{2+} channels in immature neurons and PC12 cells (Mandel *et al.*, 1988; Pollock *et al.*, 1990; Furukawa *et al.*, 1993; D'Arcangelo *et al.*, 1993; Fanger *et al.*, 1993; Fanger *et al.*, 1995; Levine *et al.*, 1995; Holm *et al.*, 1997). However, I believe that this is the first to report of a GPCR neurotransmitter regulating the expression of Ca^{2+} channels both *in vivo* and *in vitro*. Selectively cutting pre-ganglionic C-fibers to stop the synaptic release of LHRH, reduced the I_{Ba} density by ~28%, a similar percentage difference to that seen between 6d cultured neurons and 6d LHRH treated neurons from *in vitro* experiments. By cutting the preganglionic C-fibers, we selectively removed nearly all ganglionic LHRH (Jan *et al.*, 1979), without altering cholinergic B-fiber/B-cell transmission. In BFSG, LHRH is the only known transmitter able to diffuse from C-fibers to affect B-cells. Thus, it is believed that the only effect of cutting preganglionic C-fibers on B-cells was the interruption of LHRH transmission.

In other systems, activation of the Ras/ERK either directly or via agonist application leads to potentiation of Ca^{2+} current (Fitzgerald & Dolphin, 1997; Fitzgerald, 2000; Dziema & Obrietan, 2002; Fitzgerald, 2002). This effect has also been reported upon PKC stimulation (Yang & Tsien, 1993; Dziema & Obrietan, 2002). The time

course of this effect suggests that the effect is mediated by modulation of existing channels in a phosphorylation dependent manner. This raises the possibility that the effect of LHRH on I_{Ba} reported here is not due to increased channel expression, but merely the result of LHRH inducing the long-term phosphorylation of existing Ca^{2+} channels. However, this is probably unlikely for several reasons. Firstly, a 10 min application of 200 ng/ml NGF which is known to activate MAPK in BFSG (Lei *et al.*, 1998) did not cause a difference in I_{Ba} compared to the effect of a 10 min application of the MEK inhibitor U0126, which is known to reduced basal Ca^{2+} currents within the time in sensory neurons (Fitzgerald, 2000) ($n>9$ in both cases; data not shown). As the MEK inhibitor and the ERK activator produced similar effects, it appears as though Ca^{2+} channels in frog sympathetic neurons are not acutely regulated by the activation of ERK. Secondly, the long-term effect of LHRH while being sensitive to the action of the MEK inhibition, PD98059, were also sensitive to the actions of the transcription inhibitor, actinomycin-D. This suggests that the LHRH effect is due to altered channel expression. Thus the action of LHRH on Ca^{2+} channels is believed to be a neurotrophic, not neuromodulatory effect.

Conclusion

Whereas NGF up-regulation of Ca^{2+} channel expression in BFSG persists as long as target-derived neurotrophin is available, the neurotrophic effect of LHRH is more labile, as it depends on peptide release and hence neuronal activity in pre-ganglionic nerve fibers. Since neuropeptide release is favored by intense activity (Peng & Horn, 1991), regulation of Ca^{2+} channels by LHRH may couple pre-ganglionic activity to alterations in the electrical properties of pos-ganglionic cells. The release of neuropeptides from pre-ganglionic fibers causes long term increases in tyrosine hydroxylase activity and noradrenaline synthesis (McKeon & Zigmond, 1993). Due to the strong correlation between Ca^{2+} influx and neurotransmitter release, increased Ca^{2+} conductance at sympathetic postganglionic terminals may augment sympathetic outflow to target tissues. This effect may be relevant to understanding disease process such as hypertension and congestive heart failure, which involves increases in sympathetic outflow to blood vessels, the heart and other visceral organs.

Table 4-1. Effects of enzyme inhibitors on LHRH-induced I_{Ba} potentiation

Enzyme Inhibitor	**Control I_{Ba} Density (pA/pF) Blank Control**	**I_{Ba} Density in LHRH (pA/pF) Positve Control**	**I_{Ba} Density in Inhibitor (pA/pF)**	**I_{Ba} Density in LHRH + Inhibitor (pA/pF)**
Actinomycin D (0.02 μg/ml)	117.7±6.8 (49)	175.4.±16.0 (35)	104.9±7.0 (17)	105.2±6.8 (26)
α-HFA (10 μM)	117.7±6.8 (49)	175.4.±16.0 (35)	103.8±15.2 (15)	100.3±7.2 (22)
Perillic Acid (100 μM)	117.7±6.8 (49)	175.4.±16.0 (35)	61.9±4.6 (25)	58.8±3.7 (23)
PD 98059 (10 μM)	117.7±6.8 (49)	175.4.±16.0 (35)	112.1±9.9 (19)	129.3±9.1 (20)
Chelerythrine (1 μM)	117.7±6.8 (49)	175.4.±16.0 (35)	74.0±4.0 (28)	90.8±9.2 (25)
U73122 (20 μM)	67.2±7.3 (23) a	116.8±16.4 (19) a	63.4±4.7 (25)	67.3±5.3 (26)
H-89 (1 μM)	117.7±6.8 (49)	175.4.±16.0 (35)	122.6±10.2 (21)	96.0±4.9 (27)
PP1 (1.5 μM)	117.7±6.8 (49)	175.4.±16.0 (35)	44.1±2.5 (23)	94.0±10.1 (23) *
Wortmannin (100 nM)	117.7±6.8 (49)	175.4.±16.0 (35)	48.3±6.2 (18)	72.1±8.4 (17) *

Data are expressed as means±SE with n-values in parentheses; statistical tests were performed with one-way ANOVA. Current densites are maximum values recorded at 0mV. (a) Control values for U73122 were obtained with the inactive analogue U73343 (20 μM). All other data for the blank and positive controls were pooled and obtained from cells in serum free defined medium. Inhibitors: Actinomycin-D, inhibitor of DNA transcription. α-HFA, and perrilic acid, inhibitors of Ras. PD-98059, inhibitor of MEK. Chelerythrine, inhibitor of PKC. U73122, inhibitor of PLC. H-89, inhibitor of PKA. PP1, inhibitor of Src kinases. Wortmannin, inhibitor of PI3 Kinase. * signify $P<0.05$ of inhibitor with LHRH v.s. inhibitor alone.

Figure 4-1. Typical Ca^{2+} channel currents (I_{Ba}) recorded from an acutely dissociated BFSG B-cell (A), a B-cell cultured for 6 days in control media without (B), and with LHRH (C). Neurons were kept at a holding potential of –9 0mV. Families of Ba^{2+} currents were evoked by a series of depolarizing voltage commands (30 ms pulse length, 10 mV per step); tail currents were recorded at –40 mV. Voltage recording shown is associated with I_{Ba} of the cell cultured for 6 d in control media.

Figure 4-1

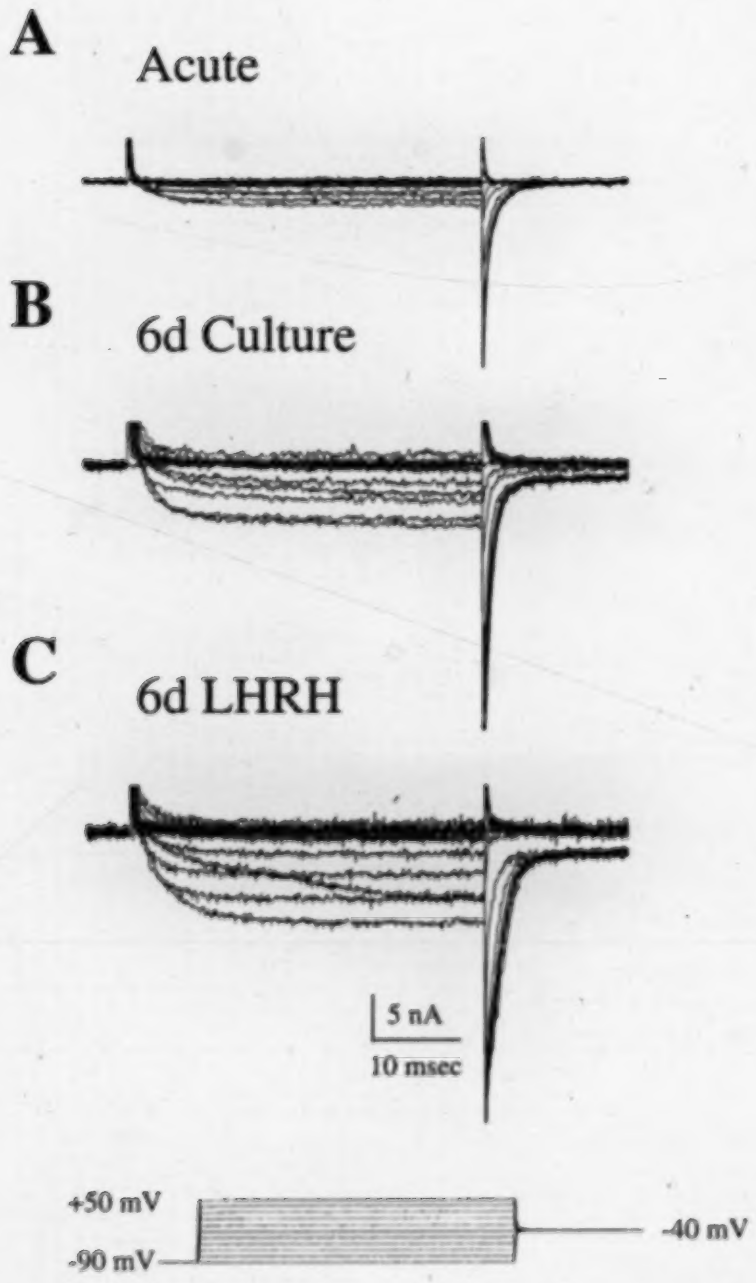


Figure 4-2. Quantified effect of LHRH on I_{Ba}. Peak (total) current **(A)**, current density input capacitance (C_{in}) **(B)**, and peak current density **(C)** recorded from acutely dissociated cells and from cells maintained in culture for 6 days in the presence or absence of LHRH. Asterisks indicate significance at the level of $p<0.05$ between columns.

Figure 4-2

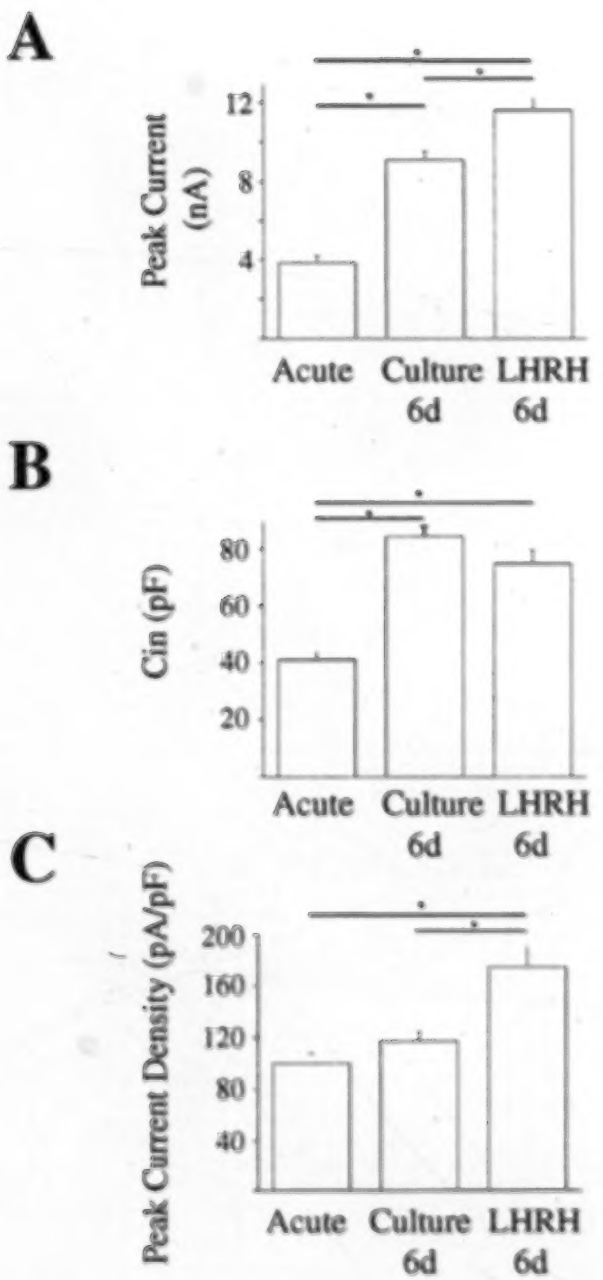

A
Peak Current (nA)
12
8
4
Acute
Culture 6d
LHRH 6d
B
Cin (pF)
80
60
40
20
Acute
Culture 6d
LHRH 6d
C
Peak Current Density (pA/pF)
200
160
120
80
40
Acute
Culture 6d
LHRH 6d

Figure 4-3. Ba^{2+} current-voltage relationships showing the effect of culturing BFSG B-neurons in the absence or presence of LHRH for 6 d. Current-density voltage plots for I_{Ba} from acutely dissociated **(A)**, 6d control cultured **(B)**, and 6 d cultured neurons in the presence of LHRH **(C)**. Three different holding potentials were used (V_h: -90, -60, -40 mV). N>20 for all data points shown. Error bars not visible when less than the symbols used to designate the data points.

Figure 4-3

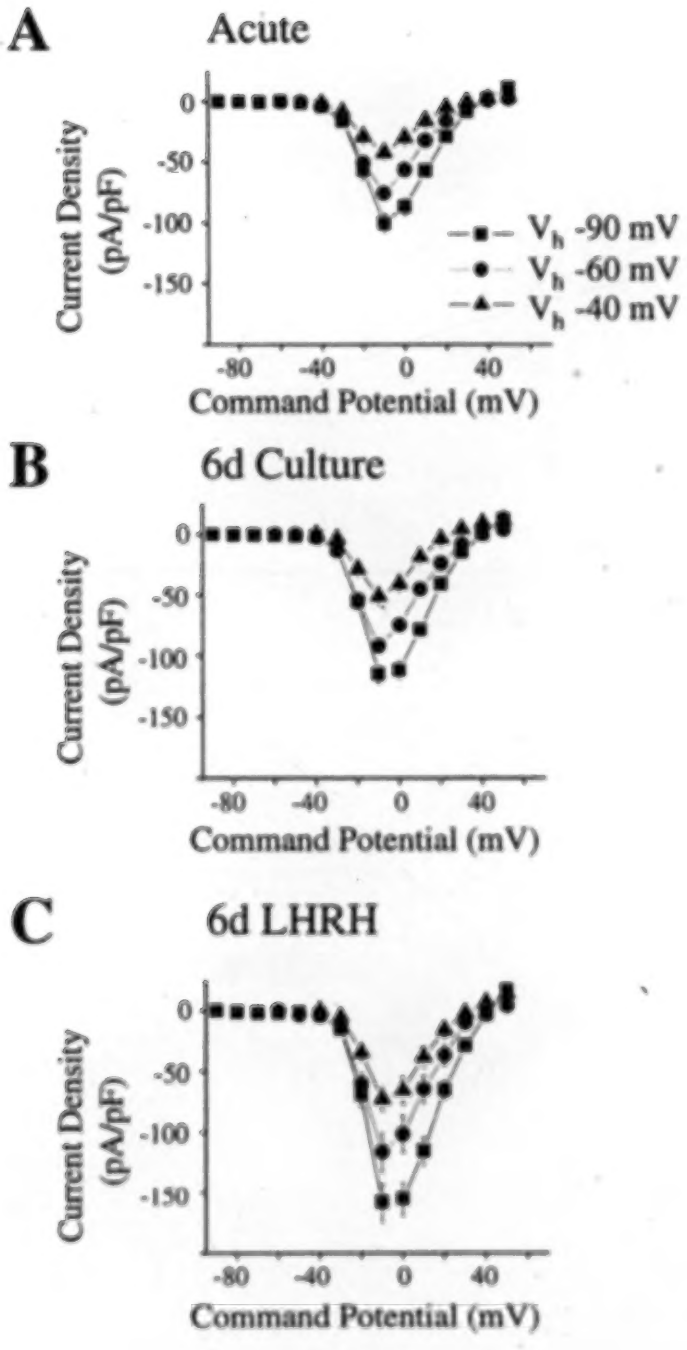

A
Acute
0
-50
-100
-150
Current Density (pA/pF)
-80
-40
0
40
Command Potential (mV)
V_h -90 mV
V_h -60 mV
V_h -40 mV
B
6d Culture
0
-50
-100
-150
Current Density (pA/pF)
-80
-40
0
40
Command Potential (mV)
C
6d LHRH
0
-50
-100
-150
Current Density (pA/pF)
-80
-40
0
40
Command Potential (mV)

Figure 4-4. Effects of LHRH on voltage dependence of I_{Na} activation and inactivation, **(A)** Activation curves for normalized g_{Na} for acutely dissociated cells, and cells cultured in the presence or absence of LHRH. I_{Na} was evoked by a series of 20 ms command potentials. Tail currents at the end of each pulse was recorded at –40mV. Tail current amplitudes were estimated by fitting the data to a monoexponential function and extrapolating the amplitude at zero time. The solid line represents a plot fitted to a Boltzmann equation:

$$g/g_{max} = 1/[1+\exp((V_{1/2}-V)/V_s)]^2$$

Where ($V_{1/2}$) is the mid-point voltage (-4.7mV) and (V_s) is the steepness factor (10.5 mV) (Sala, 1991). **(B)** Normalized isochronic inactiation curves for the three experimental conditions. I_{Na} was evoked at 0mv from various holding potentials. N>20 for all data in both figures.

Figure 4-4

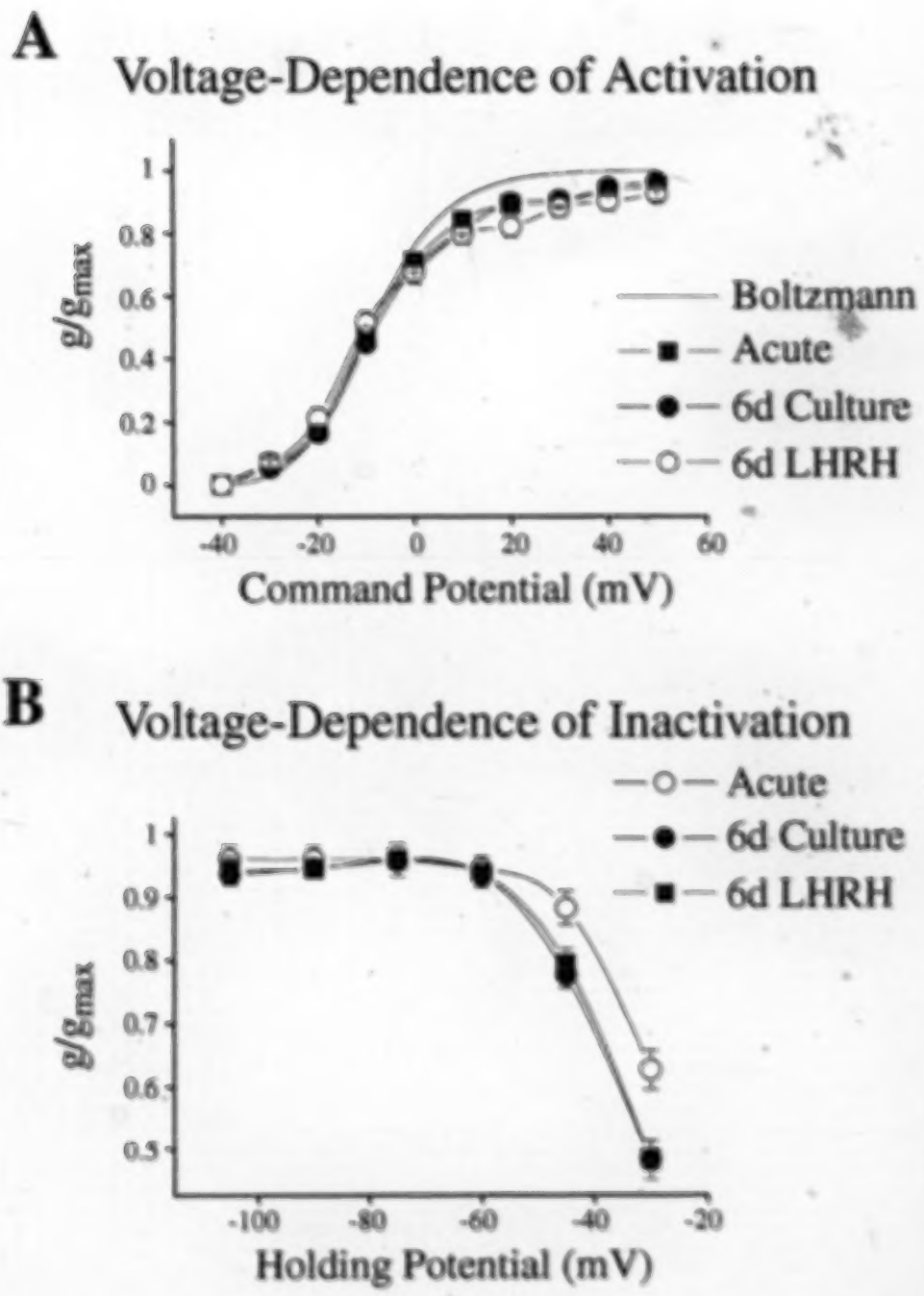

A
Voltage-Dependence of Activation
g/gmax
1
0.8
0.6
0.4
0.2
0
-40
-20
0
20
40
60
Command Potential (mV)
Boltzmann
Acute
6d Culture
6d LHRH
B
Voltage-Dependence of Inactivation
Acute
6d Culture
6d LHRH
g/gmax
1
0.9
0.8
0.7
0.6
0.5
-100
-80
-60
-40
-20
Holding Potential (mV)

Figure 4-5. LHRH receptors desensitized by culturing BFSG B-neurons for 6d in the presence of LHRH. **(A)** Suppression of I_{Ba} by acute application of 1 μM LHRH on acutely dissociated cells and cells cultured in the presence and absence of LHRH for 6d. **(B)** Suppression of I_{Ba} by acute application of 100 μM NA on cells cultured in the presence and absence of LHRH for 6 d. Note the lack of effect of 6 d LHRH treatment on I_{Ba} suppression by NA.

Figure 4-5

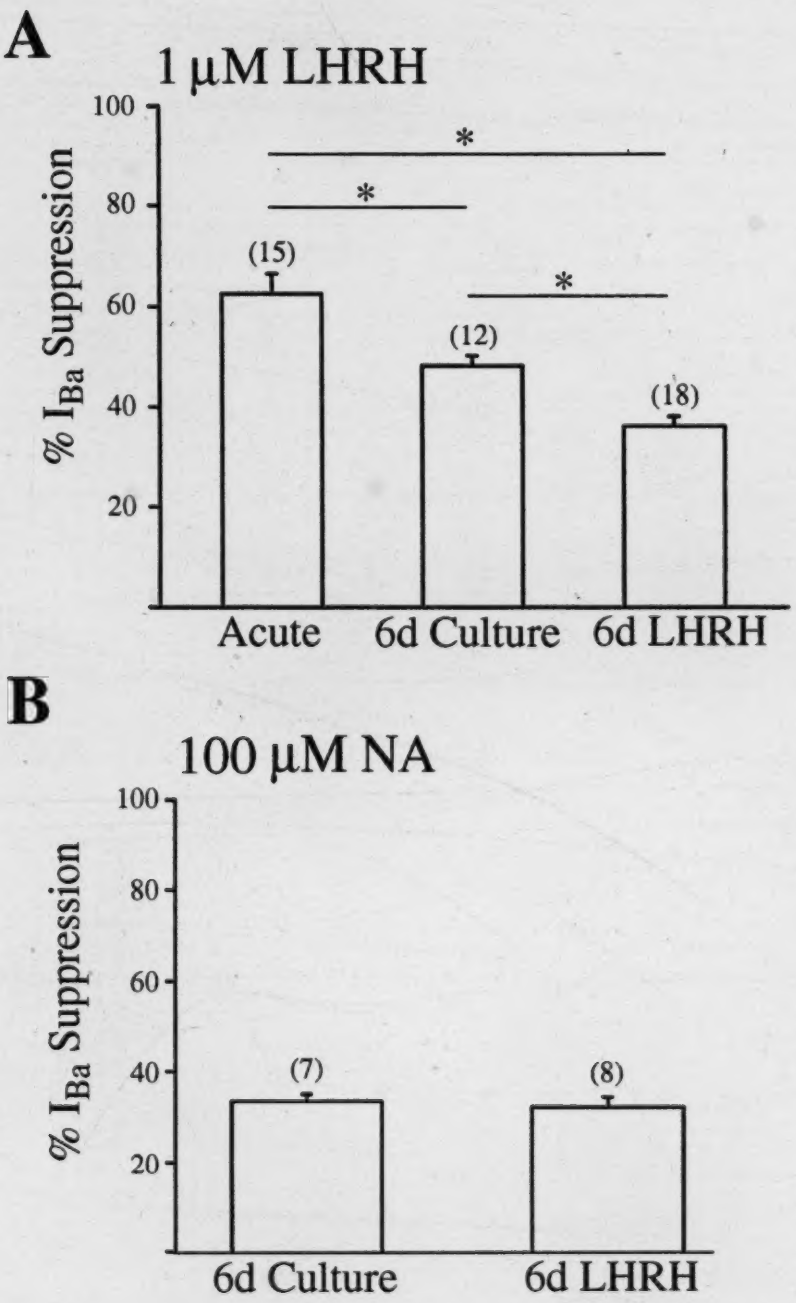

A
1 μM LHRH
% I_{Ba} Suppression
100
80
60
40
20
*
*
*
(15)
(12)
(18)
Acute
6d Culture
6d LHRH
B
100 μM NA
% I_{Ba} Suppression
100
80
60
40
20
(7)
(8)
6d Culture
6d LHRH

Figure 4-6. Percentage of ω-Conotoxin GVIA (300 nM) sensitive $I_{Ba,N}$, percentage of nifedipine (1 μM) sensitive $I_{Ba,L}$, and percentage of ω-Conotoxin GVIA/ nifedipine-resistant $I_{Ba,\ other}$ in cells cultured in the presence or absence of LHRH. ω-Conotoxin GVIA was applied initially for 45 min to allow for full $I_{Ba,N}$ suppression to occur, followed by 15 min of nifedipine. $I_{Ba,other}$ was any residual current remaining.

Figure 4-6

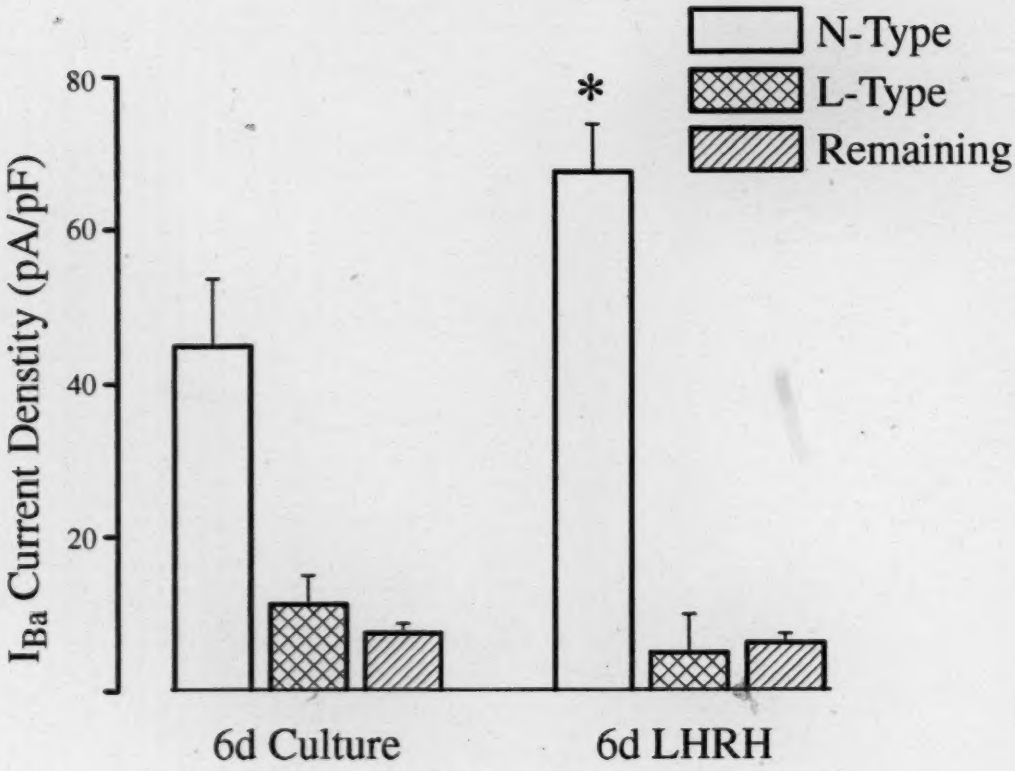

N-Type
L-Type
Remaining
80
60
40
20
I_{Ba} Current Denstity (pA/pF)
*
6d Culture
6d LHRH

Figure 4-7. Cutting preganglionic C-fiber axons to prevent the release of LHRH at C-fiber terminals reduces B-cell I_{Ba}. (A)-(B): Diagrams of experimental situations. (C): Typical Ba^{2+} currents recorded at V_h= -90 mV, from an acutely dissociated B-cell derived from a ganglia where the preganglionic C-fibers had not been cut. (D): Typical control Ba^{2+} currents recorded from an acutely dissociated B-cell derived from ganglia that had the preganglionic C-fibers cut 10d prior. (E): Peak I_{Ba} density voltage relationships for B-neurons derived from ganglia where the preganglionic C-fibers had been either cut 10d prior or had been left intact, $n \geq 17$ for both groups.

Figure 4-7

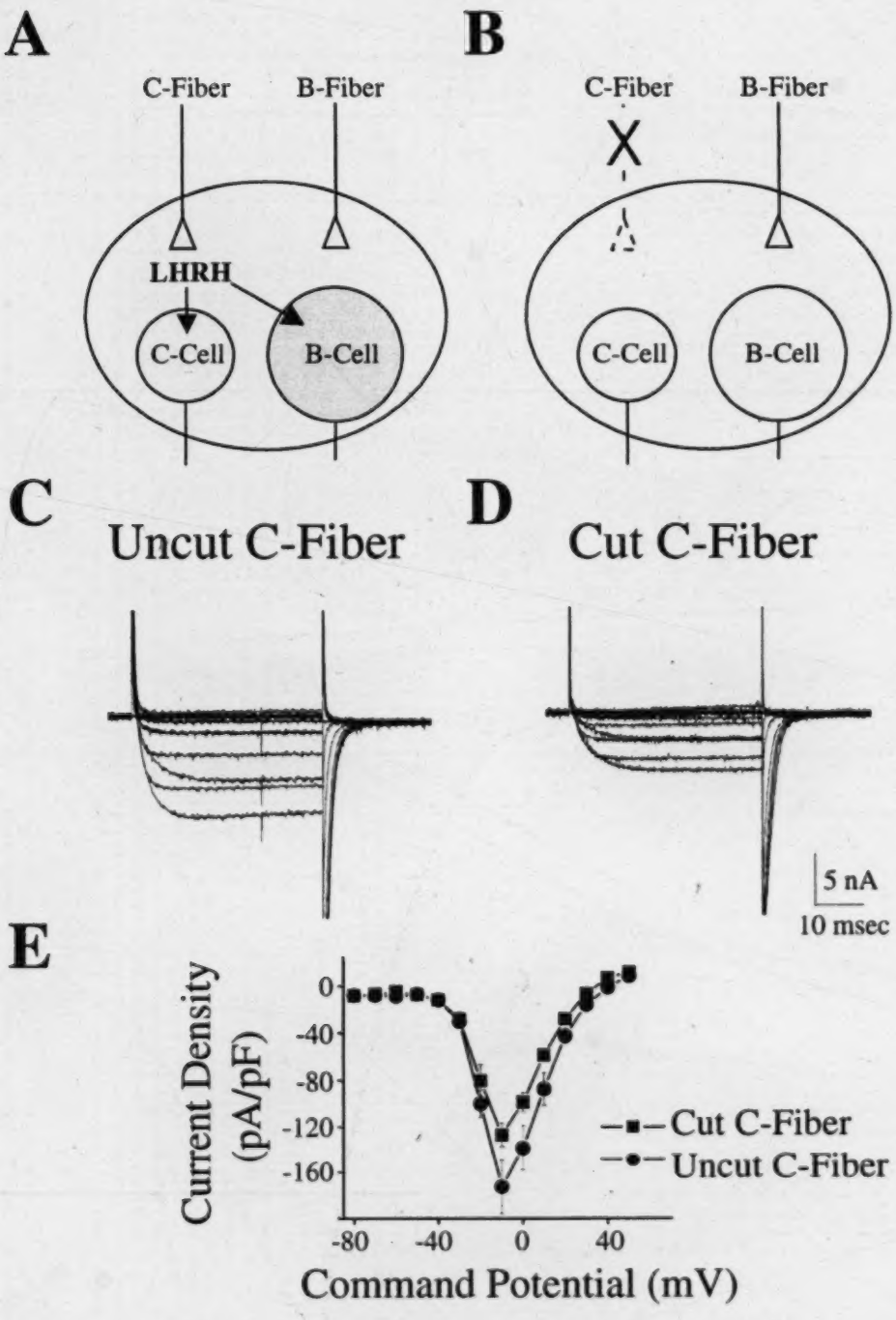

A
B
C-Fiber
B-Fiber
LHRH
C-Cell
B-Cell
C-Fiber
B-Fiber
C-Cell
B-Cell
C
Uncut C-Fiber
D
Cut C-Fiber
5 nA
10 msec
E
Current Density (pA/pF)
0
-40
-80
-120
-160
-80
-40
0
40
Command Potential (mV)
Cut C-Fiber
Uncut C-Fiber

Figure 4-8. Transcriptional blocker Actinomycin D (0.02 μg/ml) blocked the enhancement of I_{Ba} in response to LHRH. Currents evoked by a series of depolarizations (10 mV interval) from the holding potential of -90 mV.

Figure 4-8

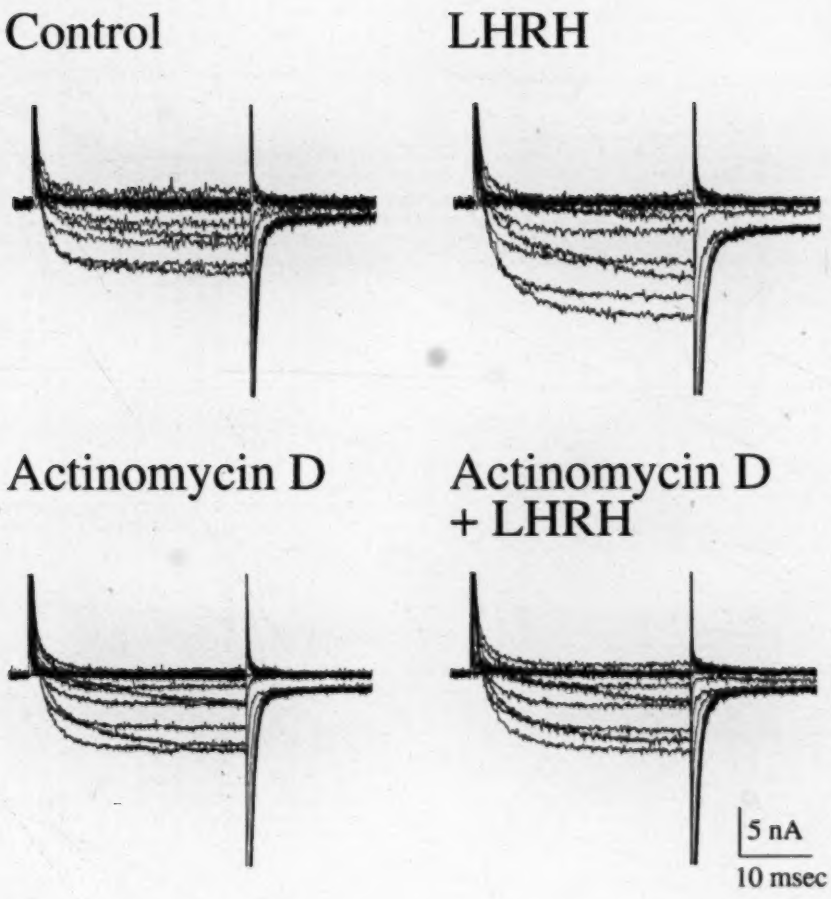


Figure 4-9. Post-translational Ras inhibitors α-Hydroxyfarnesylphosphonic acid (α-HFA) (10 μM) and perillic acid (PA) (100 μM) and the MEK inhibitor PD98059 (10 μM) blocked enhancement of I_{Ba} in response to LHRH. Currents evoked by a series of depolarizations (10 mV interval) from the holding potential of -90 mV.

Figure 4-9

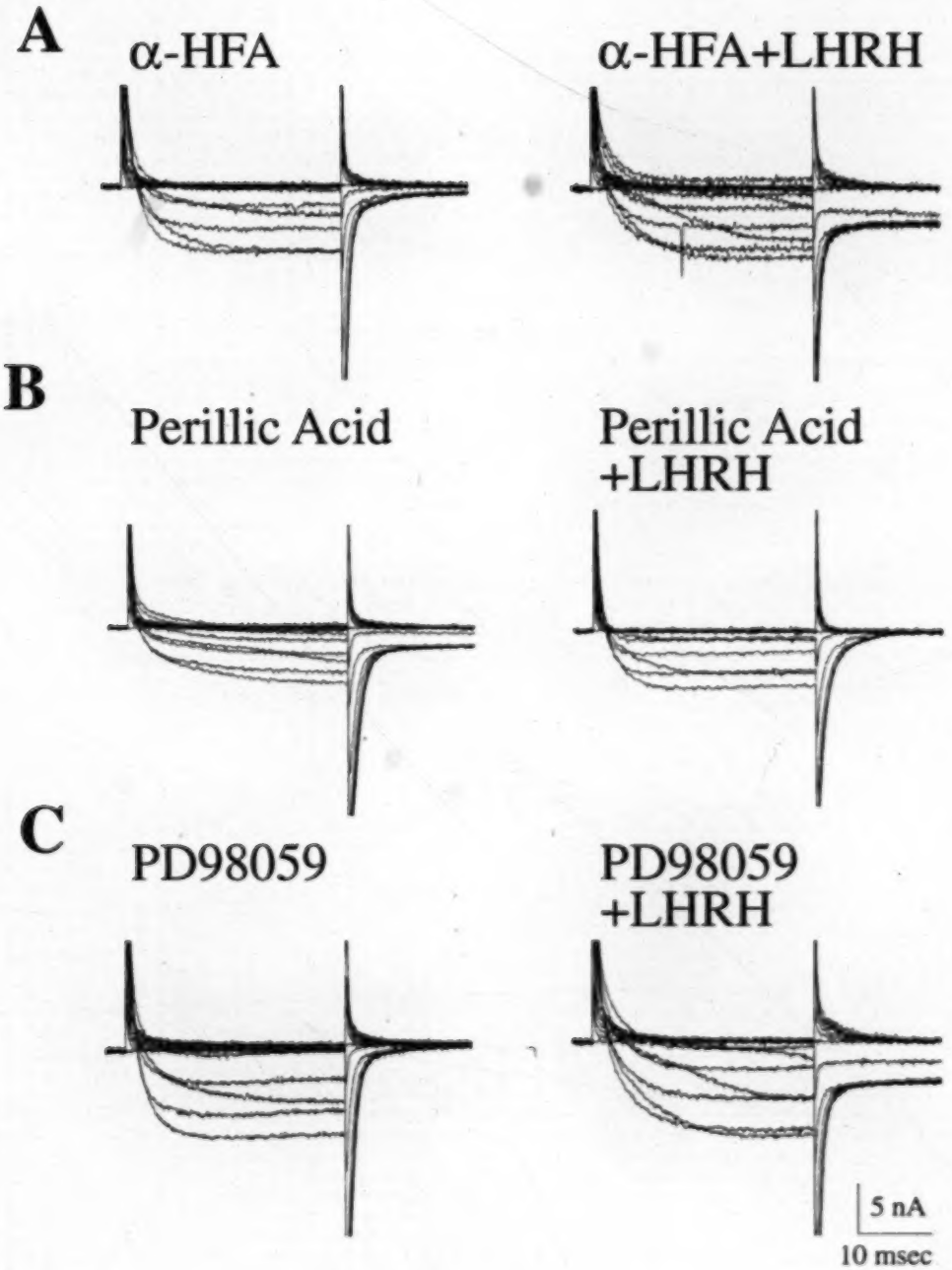

A
α-HFA
α-HFA+LHRH
B
Perillic Acid
Perillic Acid
+LHRH
C
PD98059
PD98059
+LHRH
5 nA
10 msec

Figure 4-10. Inhibition of PKC with chelerythrine (1 μM), PLC with U73122 (20 μM) and PKA with H-89 (1 μM) blocked enhancement of I_{Ba} in response to LHRH. Note the lack of effect of the inactive analogue of U73122, U73343 (20 μM) in preventing LHRH-mediated I_{Ba} potentiation. Currents evoked by a series of depolarizations (10 mV interval) from the holding potential of -90 mV.

Figure 4-10

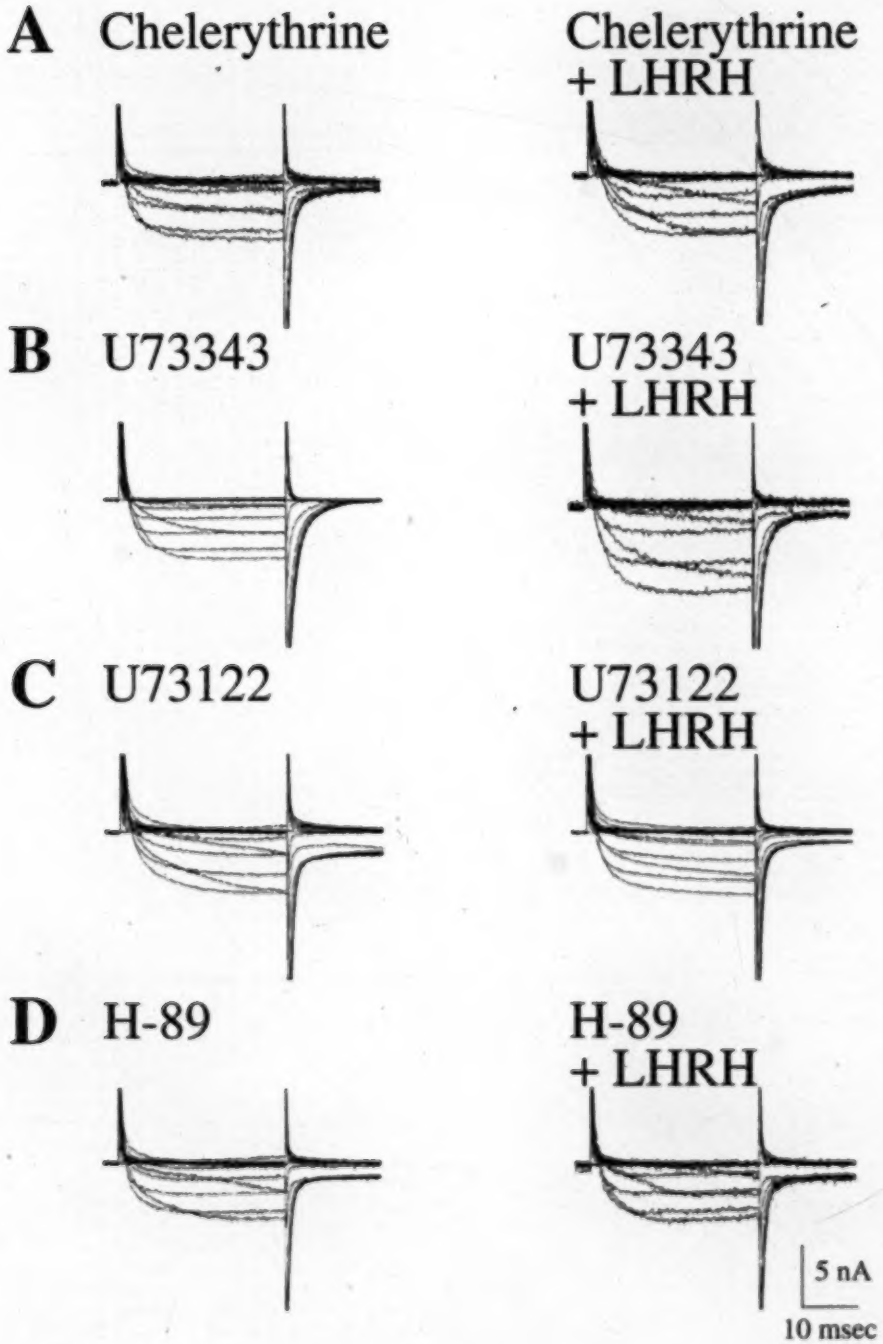

A
Chelerythrine
Chelerythrine
+ LHRH
B
U73343
U73343
+ LHRH
C
U73122
U73122
+ LHRH
D
H-89
H-89
+ LHRH
5 nA
10 msec

Figure 4-11. Lack of effect of the inhibition of Src with PP1 (1.5 μM) or PI3K with wortmannin (100 nM) on the enhancement of I_{Ba} in response to LHRH. Currents evoked by a series of depolarizations (10 mV interval) from the holding potential of -90 mV.

Figure 4-11

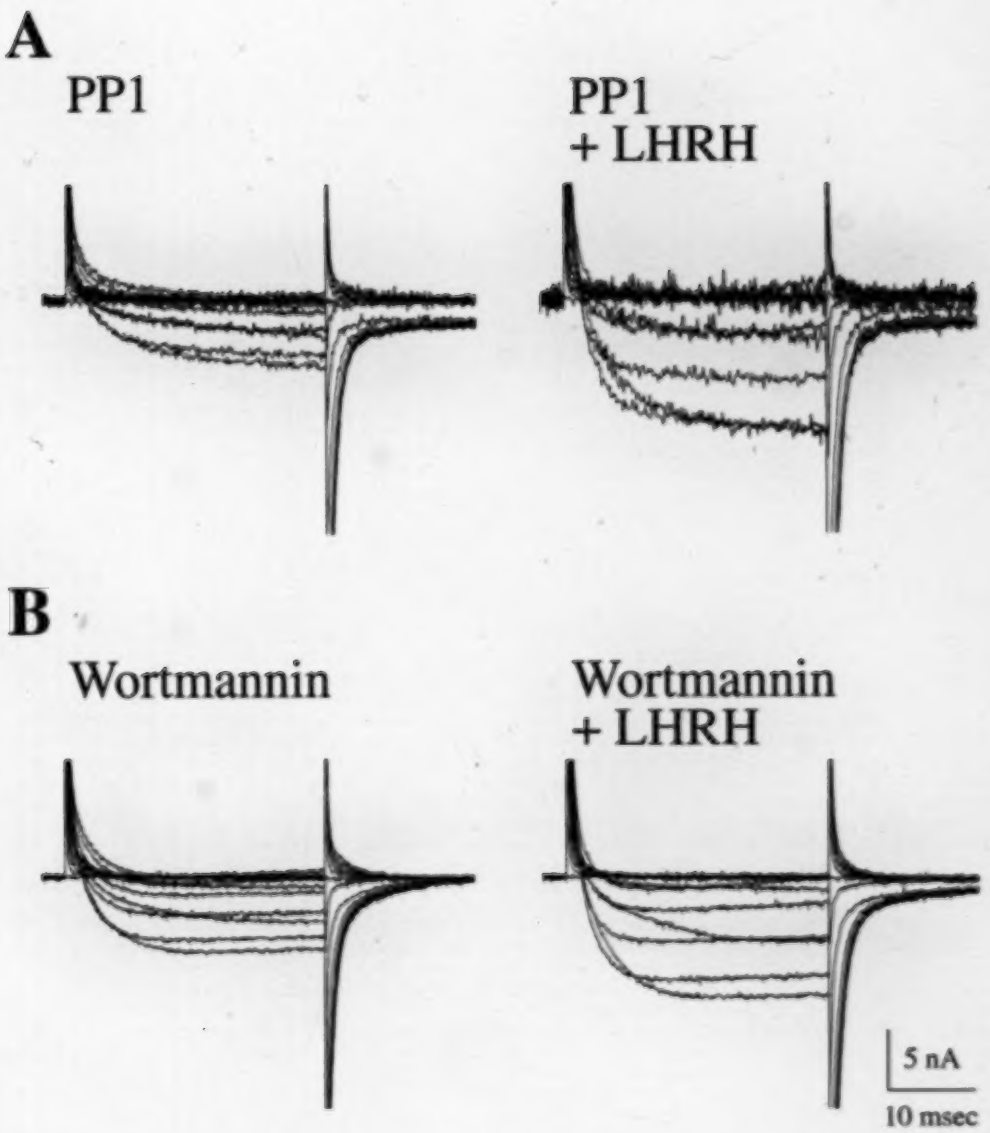


Figure 4-12. Culturing B-neurons for 6d in the presence of the PKC activator PMA (80 nM) increases peak I_{Ba}, an effect which is not seen with the inactive analogue 4α-Phorbol (80 nM). Currents evoked by a series of depolarizations (10 mV interval) from the holding potential of -90 mV.

Figure 4-12

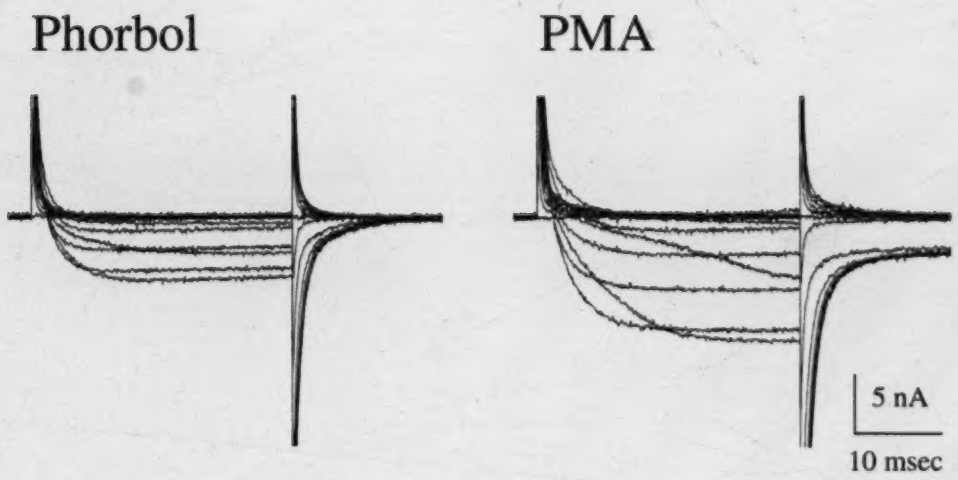


Figure 4-13. Activation of protein kinase C (PKC) by PMA (80 nM), but not protein kinase A (PKA) by Sp-cAMPS (10 μM) mimics the effects of LHRH in potentiating I_{Ba}. Current densities are maximum values recorded at 0mV. Asterisks indicate significance at the level of $p<0.05$ between columns. Values in parenthesis are n-values for each group. As controls, phorbol (80 nM) and Rp-cAMPS (10 μM) were used at similar concentration to the active agents.

Figure 4-13

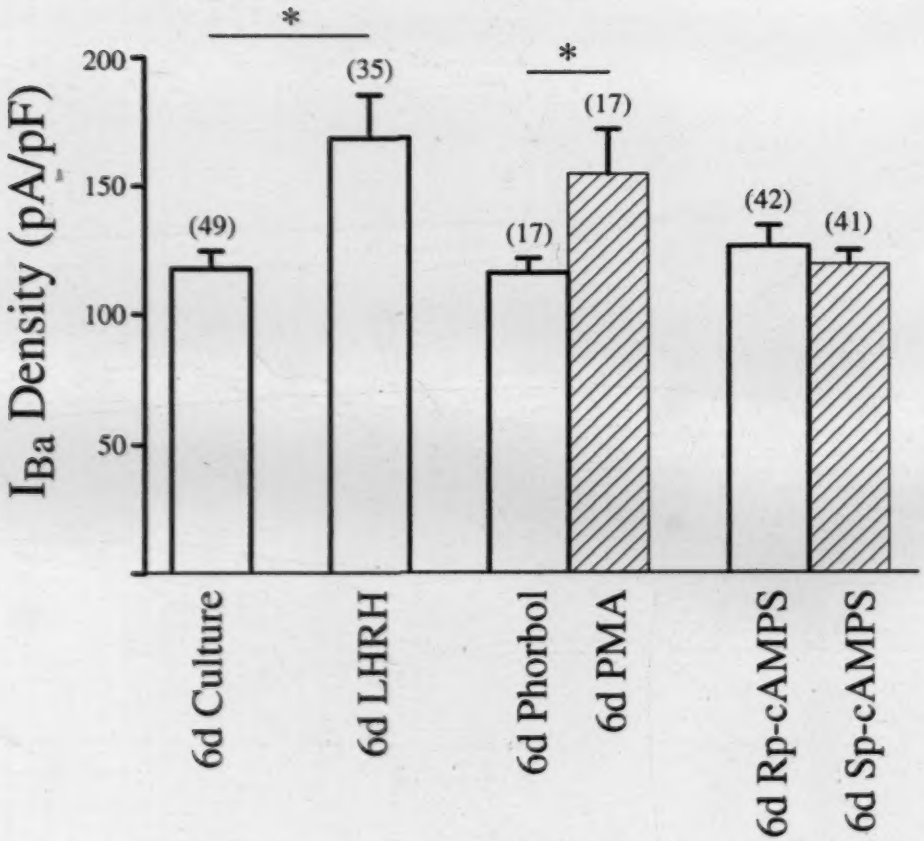
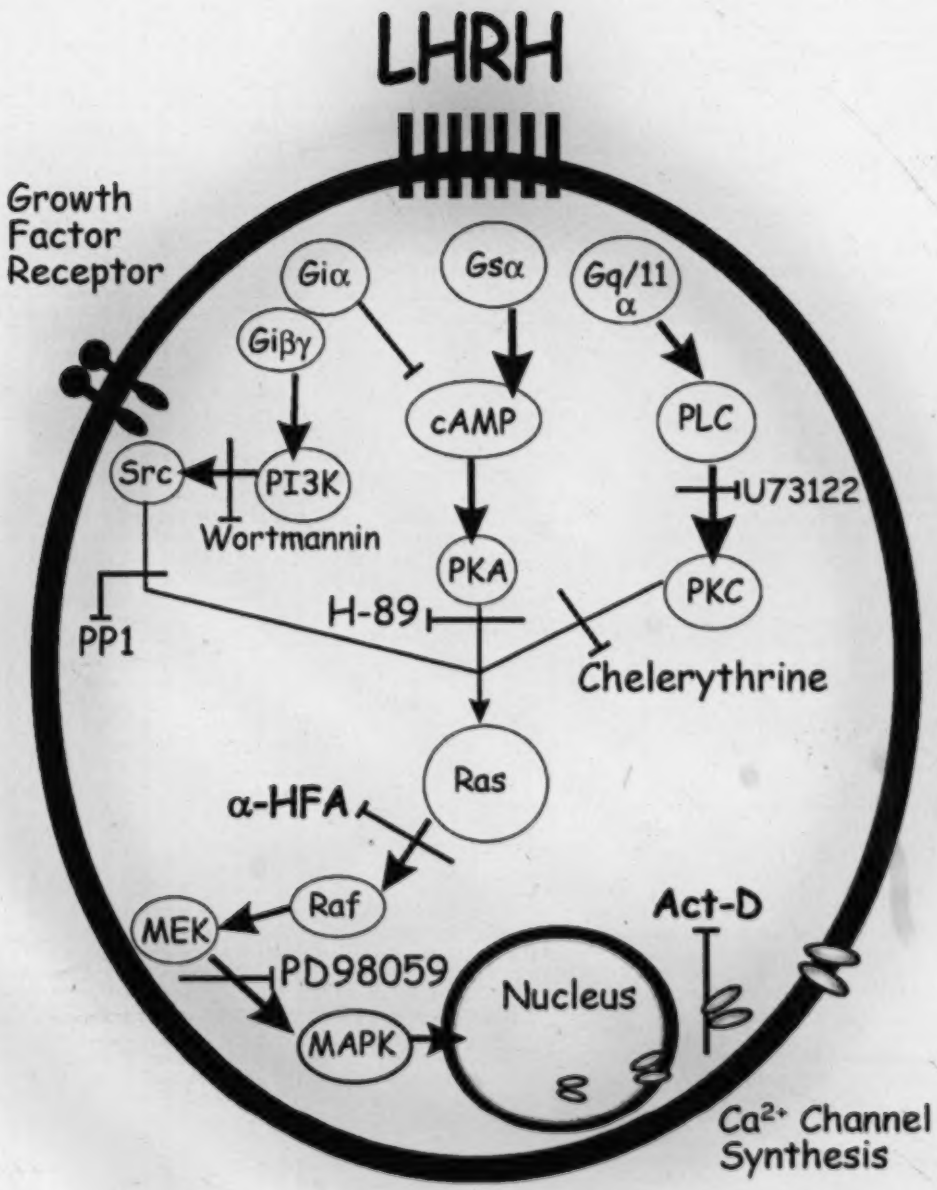

*
*
200
150
100
50
I_{Ba} Density (pA/pF)
(49)
(35)
(17)
(17)
(42)
(41)
6d Culture
6d LHRH
6d Phorbol
6d PMA
6d Rp-cAMPS
6d Sp-cAMPS

Figure 4-14. Diagram of the possible signaling pathways utilized by the LHRH-R in BFSG in mediating the increase in Ca^{2+} channel synthesis.

Figure 4-14


LHRH
Growth Factor Receptor
Giα
Gsα
Gq/11 α
Giβγ
cAMP
PLC
Src
PI3K
U73122
Wortmannin
PKA
PKC
H-89
PP1
Chelerythrine
Ras
α-HFA
Raf
MEK
Act-D
PD98059
Nucleus
MAPK
Ca2+ Channel
Synthesis

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Crowell, P. L., Chang, R. R., Ren, Z. B., Elson, C. E., & Gould, M. N. (1991). Selective inhibition of isoprenylation of 21-26-kDa proteins by the anticarcinogen d-limonene and its metabolites. *Journal of Biological Chemistry* **266**, 17679-17685.

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CHAPTER 5

GENERAL DISCUSSION

In this thesis, both the *short-term* and *long-term* regulation of voltage-dependent ion channels by neurotransmitters in adult sympathetic post-ganglionic neurons has been examined. The experiments examined the mechanisms involved in the acute suppression of M-type K^+ channels by ATP, muscarine and LHRH, and the *long-term* regulation of Ca^{2+} channel expression by LHRH. Investigation of the actions of the same neurotransmitter on ionic conductances over the course of both seconds and days advances the understanding of the mechanisms by which sympathetic neurotransmitters function.

This chapter will review the results and conclusions from the preceding three chapters and will then connect the findings to provide an overview to address general issues regarding the mechanisms of action of neurotransmitters on ion channels in the sympathetic nervous system.

Chapter Summaries

The results from chapters 2 and 3 have shown that agonist-induced g_M suppression in BFSG is disrupted by the perturbation of lipid and inositol phosphate cycles, as would be predicted if the PLC-mediated depletion of PIP_2 is the signal for channel closure.

Chapter 2 examined the signal transduction of agonist-induced M-channel suppression. The actions of ATP and muscarine were examined. The principal findings were: (1) PIP_2 re-synthesis was required for recovery of g_M after ATP- or muscarine-induced suppression; (2) disruption of PIP_2-PLC interactions reduced ATP- and muscarine- induced g_M-suppression (3) addition of PIP_2 slowed the rundown of expressed KCNQ2/3 channels and had a modest potentiating effect on native M-channels (4) disruption of PIP_2-channel interactions suppressed KCNQ2/3 current. These findings can be explained in terms of the 'PIP_2 depletion hypothesis' for agonist-induced M-channel suppression (Stemkowski *et al.*, 2002; Suh & Hille, 2002; Zhang *et al.*, 2003), which states that M-channels are maintained in an open configuration by the presence of plasma membrane PIP_2. ATP- and muscarine-induced activation of PLC mediates the hydrolysis of PIP_2. The result is a loss of plasma membrane PIP_2 thus promoting M-channels to assume a closed configuration (i.e. g_M suppression).

Two separate mechanisms have been proposed for agonist-induced M-channel suppression: (1) Muscarine and nucleotides (as examined in amphibian neurons) mediate suppression of M-channels via signaling 'upstream' from PLC, involving depletion of PIP_2 (Suh & Hille, 2002; Zhang *et al.*, 2003; see chapter 2), and (2) bradykinin and ATP in rat SCG neurons suppress g_M via the IP_3-mediated release of Ca^{2+} from intracellular stores (Cruzblanca *et al.*, 1998; Bofill-Cardona *et al.*, 2000). Chapter 3 examined which of these two pathways mediates LHRH-induced g_M suppression. The principal findings were: (1) LHRH mediates suppression of g_M via PLC but not by 'downstream' signaling involving IP_3, Ca^{2+}, or PKC; (2) PIP_2 re-synthesis was required for recovery of g_M after LHRH-induced suppression; (3) sequestration of PIP_2 reduced steady state g_M and reduced LHRH-mediated suppression of g_M. Thus, like muscarine and ATP, but unlike bradykinin, LHRH mediated M-channel suppression is best explained in terms of the PIP_2 depletion hypothesis (Suh & Hille, 2002; Zhang *et al.*, 2003). In the frog sympathetic system, it appears as though the common mechanism for inducing M-channel suppression is via the depletion of PIP_2. Thus although many agonists which mediate g_M suppression also produce increases in IP_3, Ca^{2+}, and activate PKC (Pfaffinger *et al.*, 1988; Bosma & Hille, 1989; Selyanko *et al.*, 1990; Beech *et al.*, 1991; del Rio *et al.*, 1999), these molecules are not primarily responsible for M-channels closure.

As well as being a classical slow neurotransmitter within the sympathetic system of the frog, LHRH can also activate the Ras/MAPK pathway in certain systems. Since NGF regulates ion channel expression in BFSG by this pathway it was hypothesized that LHRH may also function as a trophic factor. Chapter 4 investigated this hypothesis, specifically examining if LHRH could regulate the expression Ca^{2+} channels. The principal findings were (1) LHRH regulated the expression of Ca^{2+} channels in a transcription-dependent manner; (2) LHRH regulated Ca^{2+} channel expression both *in vitro*, and *in vivo*; and (3) this process is mediated by PLC, PKA, PKC and the Ras/MAPK pathway. As discussed above, in BFSG, LHRH mediates the late-slow e.p.s.p. The importance of this section of the thesis is that it documents how, in addition to the action of LHRH as a 'slow' neurotransmitter (Jan et al., 1979), LHRH also acts as a neurotrophic factor to exert long-term, control of Ca^{2+} channel expression in a sympathetic ganglion.

M-type K^+ Channel Regulation by PIP_2

While there is good evidence that agonist-meditated suppression of KCNQ2/3 channels in oocytes and CHO cells involves the depletion of PIP_2 (Zhang *et al.*, 2003), definitive proof of this hypothesis in a native neuron is not available. Over the course of the work for this thesis, Zhang et al. (2003) reported activation of M-channels in excised patches from SCG neurons by direct application of PIP_2. However, in those experiments, the holding potential was at a positive potential sufficient to activate a variety of other conductances. Due to the recording techniques employed it is not known if a 'true' g_M was examined. One of the difficulties faced in this thesis was the poor efficacy of PIP_2 in modulating M-channels in neurons. This was in contrast to the strong effect of PIP_2 on expressed KCNQ2/3 channels. The majority of studies examining the actions of PIP_2 in modulating ion channels have been performed in expression systems, (Huang *et al.*, 1998; Xie *et al.*, 1999; Zhang *et al.*, 1999; Bian *et al.*, 2001; Runnels *et al.*, 2002; Zhang *et al.*, 2003) and few reports document modulation of native channels by PIP_2 (Fan & Makielski, 1997; Zhang *et al.*, 2003). This suggests that channels in native neurons are more difficult to modulate than when they are exogenously expressed. The reason for this difficulty is unknown. One possibility is that in native systems exogenously applied PIP_2 does not insert into the correct neuronal membrane. Alternatively, native channels may be complexed with other signalling proteins, a situation which may not occur with expressed channels, and may prevent PIP_2 from obtaining the correct conformation with the channels. KCNQ2 channels have been found to form a complex with A-kinase anchoring protein 150 (AKAP 150) and PKC (Hoshi *et al.*, 2003), suggesting that such a protein complex may occur. The possiblility that this complex assembles in native neurons, but does not when KCNQ2/3 channels are expressed in tsA-201 or COS-1 cells may explain the effect of exogenous PIP_2 when tested in the expression systems, but not BFSG neurons (see Chapter 2). In the field of signal transduction, as well as ion channel research, it is becoming increasingly apparent that signaling is regulated by scaffold proteins. These structural determinants are important not only in signaling themselves, but also in assembling complexes of the correct molecules in the appropriate locations. Thus the emerging concept is that enzymes do not function in isolation. Future studies

examining the regulation of ion channels will undoubtedly focus on these macro-molecular complexes and not on only their single protein components.

An additional factor which may explain the lack of effect of PIP_2 on native I_M may be current density. In native neurons, the current density (taken to be the number of channels per area of membrane) is substantially less than the current density of tsA-201 and COS-1 cells. Approximate calculations show that there was approximately a four times greater density of M-channels in tsA-201 and COS-1 cells compared to native neurons. By transfecting these mammalian cells to express KCNQ2/3 channels it was possible to 'drive' the expression to higher levels than are naturally present in native BFSG neurons. Thus in the expression systems, there is a higher ratio of channel to membrane. When PIP_2 was exogenously applied, KCNQ2/3 channels would have been more likely to be influenced by PIP_2 than native channels. This may partially explain the stronger effect of PIP_2 when applied to expressed channels compared with native M-channels in BFSG neurons.

The evidence from Chapters 2 and 3, support the PIP_2 depletion hypothesis as the mechanism responsible for LHRH-, ATP- and muscarine-induced g_M suppression. However, there still exists the possibility that other processes are occurring which may mediate the transduction of agonist-induced M-channel closure. The fact that DAG analogues are known to suppress I_M (Bosma & Hille, 1989; Clapp *et al.*, 1992; Selyanko *et al.*, 1992; Chen *et al.*, 1994) still leaves open the possibility that the production of DAG in parallel with the depletion of PIP_2 may also contribute to agonist-induced M-channel suppression. The evidence from Chapter 2 suggests that if there is an effect of DAG it is not meditated by DAGK implying a possible direct effect of DAG (Chen *et al.*, 1994; Clapp *et al.*, 1992).

In the work of Hoshi et al. (2003), the complex of AKAP 150, PKC and KCNQ2 channels was required for channel inhibition by muscarinic agonists. From this, it was deduced that phosphorylation of the channel by PKC promotes M-channel suppression. However, a variety of tests to inhibit PKC failed to block a substantial portion of g_M suppression induced by moderate agonist concentrations. Additionally, the effects of inhibitors were observed only after overnight treatment, an excessive length of treatment for inhibitors which are known to produce an acute effect. Both of these criticisms

suggest that at best PKC plays only a minor role in transducing the inhibitory effects of muscarine on M-channel activity. The muscarinic suppression of KCNQ2/3 channels which was sensitive to PKC inhibitors was only sensitive to inhibitors that act on the diacylglycerol binding site, but not the catalytic site of PKC. This suggests that the lack of effect of chelerythrine on agonist-induced g_M suppression (see Fig 3-4) could be due to poor accessibity of this drug to its target (Hoshi *et al.*, 2003). However, in agreement with the vast majority of previous studies (see General Introduction), this thesis has shown that the role of PKC in agonist-induced g_M suppression in the frog, is most likely minor. In BFSG B-cells dialyzed with the non-hydrolyzable analogue of ATP (AMP-PNP), LHRH was found to induced a robust suppression of g_M (see Chapter 3). If channel phosporylation was the mechanism responsible for suppression, it would be unlikely that such a robust suppression would have occurred in the absence of available phosphate donors. This finding further rules out the role of a phosphoylation-dependent mechanism for agonist-induced M-channel closure in BFSG. Similar results with non-hydrolysable ATP analogues have ruled out kinase actions in mediating the effects of muscarinic agonists on g_M suppression in SCG neurons (Suh & Hille, 2002)

Zhang et al (2003) have proposed recently that the lateral diffusion of PIP_2 within the plasma membrane can account for previous observations that the second messenger is membrane delimited (Selyanko *et al.*, 1992). The proposed mechanism is that PIP_2 hydrolysis in one area of the membrane induces lateral diffusion of PIP_2 down its concentration gradient from other areas of the membrane. These experiments were performed on the well documented ACh-regulated GIRK system. This system is believed to be analogous to the current M-channel system. However, further experiments are needed to confirm the validity of this hypothesis in agonist-induced I_M suppression. Additional experiments are also required to determine the nature by which PIP_2 maintains M-channels in an open configuration. Modulation of cation conductances via PLC-mediated depletion of PIP_2 appears to be a widespread mechanism for channel modulation, as K_{ATP}. GIRK, TRPM7, VGCC channels are all regulated by PIP_2 (Fan & Makielski, 1997; Huang *et al.*, 1998; Xie *et al.*, 1999; Zhang *et al.*, 1999; Lei *et al.*, 2001; Bian *et al.*, 2001; Meyer *et al.*, 2001; Runnels *et al.*, 2002; Wu *et al.*, 2002). If

this mechanism holds true for M-channels, it may represent the elusive second messenger responsible for M-channel closure (Stemkowski *et al.*, 2002)

Remaining Questions and Future Directions

The evidence provided has given strong support for the PIP_2 depletion hypothesis as mediating M-channel suppression. However, definitive verification of the hypothesis has not been achieved. While both here and in the work of Zhang et al. (2003) PIP_2 was shown to modulate KCNQ2/3 currents, in neither work was it shown that PIP_2 modulates KCNQ2/3 current suppression by agonist. Future experiments could be to reconstitute this pathway by expressing the M_1-muscarinic receptor in the same cells as KCNQ2/3 channels (Shapiro *et al.*, 2000). With this system, it would be possible to determine if PIP_2 regulates the suppression of KCNQ2/3 by muscarine. Likewise, evidence is also lacking as to whether the addition of PIP_2 alters agonist-induced g_M suppression in a native system. Chapter 2 attempted to answer this question by comparing the effects of ATP on g_M suppression in PIP_2 and control dialyzed neurons. However, no difference was observed between these two groups. The likely explanation for this is failure was, as discussed above, the limited access of PIP_2 to the correct binding sites on the M-channel. Until it is possible to overcome this difficulty, it will not be possible to conclusively implicate this as the mechanism of M-channel suppression.

An important question which requires investigation is the means by which PIP_2 maintains M-channels in an open configuration. PIP_2 may function to activate a signalling cascade which leads to the maintenance of g_M. An alternate, simple explanation would be that the negatively charged moieties on PIP_2 electrostatically interact with positive residues on the M-channel, and that this mechanically keeps the pore of the channel open. This effect is believed to be analogous to the electrostatic interaction of PIP_2 with K_{ATP} channels. Biochemical and electrophysiological experiments indicate that PIP_2 interacts with basic residues in the C-terminus of the K_{ir} 6.2 subunit of K_{ATP} channels (Fan and Makielski, 1997). The nature of this interaction appears to be electrostatic as the number of phosphate groups and thus the number of negative chares attached to the inositol ring is critical for the phospholipids effect (Sheng and Nicols, 1998). K_{ATP} channels have short sequences rich in basic amino acids in their

COOH termini that may provide the binding site for PIP_2 (Martin, 1998). Thus, the positive charge in these short regions on the COOH terminus may be necessary for interaction with PIP_2. In fact, PIP_2 has been shown to interact with two basic residue clusters in the COOH terminus (Shyng et al., 2000). An analogous direct interaction is also predicted to occur between PIP_2 molecules and M-channels. Zhang *et al* (2003) performed mutations that neutralized a positive charge in the C-terminal tail of KCNQ2 subunits (H328C), at a site where this interaction is believed to occur. Removal of these positive C-terminal charges abolished homomeric KCNQ2 currents and significantly reduced currents generated from KCNQ2/3 heteromers, but not their plasma membrane expression, suggesting that PIP_2 may interact with the positive charges in the C-terminal tail of M-channels to maintain these channels in the open state. KCNQ2 (H328C)/KCNQ3 hetermomers exhibited greater agonist-induced inhibition over wild type channels, suggesting that agonist-induced suppression is also dependent upon the interaction of PIP_2 with the C-terminal tail of the channel.

The use of alternate techniques in the future could aid in confirming the direct interaction of PIP_2 and channel. Once such technique would be fluorescence resonance energy transfer (FRET). By attaching flouescently labeled tags to the C-terminal tail of the channel and to PIP_2 molecules, this technique could determine if PIP_2 molecules and the channel obtain a sufficiently close association to allow for their direct interaction. With this technique it would also be possible to examine if PIP_2 can directly interact with the KCNQ2/3 channels used in the expression system, as well as M-channels from BFSG. If PIP_2 was found to interact only with expressed, and not native channels, it would explain the lack of effect of PIP_2 on M-channels observed in Chapter 2, further supporting the hypothesis that native M-channels may be in tight association with a variety of signaling proteins, which limits the access of exogenously applied PIP_2. The mechanism of how endogenous, but not exogenous PIP_2 may be able to modulate native channels is currently unknown.

Receptors for LHRH, muscarine and Substance P are known to inhibit M-channels via the activation of separate pools of identical G-proteins (Simmons & Mather, 1991). Thus, the question that arises is how do these various neurotransmitters produced different effects by acting on identical pathways? Differences in the degree to which

these different agonists modulate g_M could be most easily explained by the efficacy by which different agonists activate their cognate receptors, the efficiency by which these different receptors activate PLC, and the degree of receptor desensitization/removal of agonist from the synaptic cleft to end the signal. However future experiments are still required to validate this hypothesis.

An additional interesting observation is that PLC-mediated membrane depletion of PIP_2 appears to be a common mechanism by which a variety of different cation channels are regulated. Such channels are: GIRK, TRPM7, VGCC channels (Xie *et al.*, 1999; Runnels *et al.*, 2002; Wu *et al.*, 2002). As multiple different channels utilize apparently identical signaling mechanisms, the problem arises as to how signaling from a receptor to the desired ion channel can be properly maintained. Agonist-induced g_M suppression requires a diffusible messenger (Selyanko *et al.*, 1992). This diffusible messenger has been postulated to be the removal of PIP_2 as it follows its concentration gradient from areas depleted by PLC (Ikeda & Kammermeier, 2002; Zhang *et al.*, 2003). This suggests that the area surrounding a certain receptor is subject to PIP_2 depletion. If receptors and ion channels were randomly distributed in the plasma membrane on a neuron, it would be anticipated that agonist stimulation could cause a global suppression of all conductances that are regulated in this fashion. An alternate suggestion could be that receptors and ion channels are linked in close proximity in 'signaling rafts'. Such an arrangement would ensure correct specificity of signaling from the receptor. This could be analogous to such structures as the post-synaptic density complex found in glutmatergic synapses where receptors and ion channels are in highly concentrated signaling complexes (Sheng, 2001).

As various cation conductances are regulated in a similar manner by PIP_2, this may be a common mechanism by which multiple ion channels are regulated. Classically, at least in the field of ion channel research, the plasma membrane was viewed only as an inert electrical insulator with importance to structure and not function. This dogma is now rapidly changing. The findings presented in this thesis support the view of that the lipid membrane is indeed functionally important, regulating various processes including electrophysiological ones.

Regulation of Ca^{2+} Channel Expression by Luteinizing Hormone Releasing Hormone

In the sympathetic system, neurotransmitters are known to act in a trophic manner to regulate tyrosine hydroxylase activity, noradrenaline synthesis (McKeon & Zigmond, 1993), and dendritic growth (Drahushuk *et al.*, 2002). In the CNS, neurotransmitters have also been found to act trophically to modulate dendritic growth in a variety of cell types (May & Braas, 1995; Wilson *et al.*, 2000). Various growth factors and the activation of MAPK are known to regulate neuronal ion channels (Huang & Rane, 1994). This thesis shows that a neurotransmitter, which likely acts via a GPCR, can regulate the expression of Ca^{2+} channels both *in vivo* and *in vitro*. Thus in addition to the other known roles of neurotransmitters, the regulation of ion channel expression can be added to the list of neurotrophic actions now attributable to neurotransmitters.

Co-transmitters such as LHRH have been proposed to endow synapses with variable gain, thus functioning as a 'gain control (Horn, 1992). In BFSG, this states that the late-slow e.p.s.p. generated by LHRH functions to increase the efficacy of nicotinic transmission. In BFSG, some nicotinic e.p.s.p.s can be suprathreshold for action potential generation (Dodd & Horn, 1983a). This combined with the fact that low convergence of presynaptic innervation occurs, endows nicotinic transmission to regulate the number of pre-ganglionic action potentials to equal the number of generated post-ganglionic action potentials (i.e., the input-output for all frequencies is 1:1). LHRH, as a co-transmitter functions to transform bursts of pre-ganglionic action potentials into longer bursts of post-ganglionic action potentials (i.e., the input-output for high frequencies is 1:>1) (Dodd & Horn, 1983b; Horn, 1992). The findings of Chapter 4 further our understanding of the regulation of synaptic transmission in a defined system. The regulation of Ca^{2+} channels in a post-synaptic neuron by a neurotransmitter allows for precise control of the electrical phenotype of the neuron to be related to the level of activity occurring across that synapse. At highly active synapses, the increased level of peptide released would function to promote increased Ca^{2+} channel expression. This is important for the maintenance of correct $[Ca^{2+}]_i$, which in turn regulates neurotransmitter release. By maintaining the correct level of channels, a neuron will be able to correctly signal to target tissues. Thus, in addition to mediating communication from the pre- to

post-synaptic neurons (acting as one level of 'gain control'), the pleiotrophic effect of LHRH may additionally function to increase the efficiency of synaptic transmission across active synapses. Thus, allowing neurons to be better 'equipped' to handle increased communication across their synapses. This could then be viewed as a second level of 'gain control'. From a physiological perspective, it would be interesting to determine if the neuronal effect of LHRH are seen at the level of the end organ. If increased peptidergic transmission increases the signaling abilities of a neuron, increased end organ communication may also occur. This secondary 'gain-control' could then be an efficient neural means to allow for effective regulation of target tissues, which is the primary function of the autonomic nervous system.

As Ca^{2+} channels also mediate the activation of several transcription factors (Murphy *et al.*, 1991; Bading *et al.*, 1993; Dolmetsch *et al.*, 2001) the regulation of channels could also be important for aspects of cell regulation, growth. It is known that Ca^{2+} is important for the survival of neurons (Johnson, Jr. *et al.*, 1992; Murrell & Tolkovsky, 1993), however the regulation of Ca^{2+} channels by LHRH is most likely not important for the survival of BFSG neurons, as cells were viable when cultured in the absence of LHRH.

One practical issue relating to the experimental findings on Ca^{2+} currents should be addressed here. Although good voltage control of membrane currents could be achieved in electrically compact, freshly dissociated neurons, there was some evidence of inadequate voltage clamp in current recordings from cultured cells. This appeared as unexpected, slow activation of current at some voltages, overt distortion in the voltage recording, failure of the recorded membrane voltage to attain the command voltage within the first 1-2 msec, variations in the time constants of tail currents recorded at the same voltage, and failure of the current to settle to the original baseline following termination of a voltage command. A few cells were excluded from the analysis as they displayed unclamped Ca^{2+} spikes in response to voltage commands or they displayed tail currents that could not be fit by a mono-exponential time course. In terms of activation curves (derived from tail currents), the inadequacy of the clamp conditions would preclude a detailed, quantitative analysis of the activation kinetics. However the main point of this study was to look at current amplitude. In this regard, the above mentioned

clamp inadequacies would have been a systematic error (affecting both 6d culture, and 6d LHRH treated neurons). This same bias would thus be present in both groups and thus would unlikely have affected the overall conclusion from this study examining the chronic effects of LHRH of I_{Ca} amplitude.

Future Directions

LHRH most likely mediates the up-regulation of Ca^{2+} channels via activation of PLC, PKA and PKC leading to the activation of the Ras/MAPK pathway (see chapter 3). The degree of certainty that can be placed upon this finding is limited as only a pharmacological approach was taken to probe the signal transduction pathway. Using alternative techniques in parallel to the use of enzyme inhibitors would have strengthened the argument that this is the pathway utilized by the LHRH receptor. One such technique would be Western blotting to identify whether certain proteins are phosphorylated upon LHRH stimulation. However, this technique identifies only the pathways stimulated by LHRH, and not those which are involved in regulation of Ca^{2+} channel expression. This technique is now ongoing in the lab, and preliminary evidence has determined that LHRH leads to phosphorylation or ERK (thus activating it), but does not lead to the activation of TrkA. This indicates that most likely the LHRH receptor does not transduce its signal via the transactivation of the NGF receptor. Future studies looking to re-examine this pathway could make use of existing molecular biology techniques to inhibit various aspects of the transduction mechanism to map the pathways involved. One technique which could be used would be RNA interference (RNAi). With this approach it would be possible to induce post-translational silencing of selected genes (Fire *et al.*, 1998). Blocking the expression of certain pathway proteins to determine their involvement in the LHRH-induced regulation of Ca^{2+} channels would provide a complimentary approach to the pharmacological experiments described in Chapter 4. This technique has been recently shown to be one that is amenable to use with neurons (Krichevsky & Kosik, 2002), suggesting that it may be a viable means to further elucidate the signal transduction pathway utilized by LHRH in mediating the up-regulation of Ca^{2+} channels in BFSG.

Conclusions

The commonality among sections of this thesis is the role played by GPCRs in regulating various aspects of ion channel function. GPCRs signal through a variety of different pathways to regulate multiple different effectors. An apparent second factor common in all aspects of this thesis is PLC. As documented in previous chapters, this enzyme is necessary for the acute suppression of M-channels and the tropic regulation of Ca^{2+} channel expression by GPCRs. This finding illustrates the pleurotophic signaling of not only a single receptor, but also a single enzyme effector in mediating different effects along vastly different time courses.

As opposed to their well-defined classical long acting roles, it is now known that neurotrophins are able to act on time courses of seconds to minutes to modulate synaptic responses (Lohof *et al.*, 1993; Figurov *et al.*, 1996; Stoop & Poo, 1996; McAllister *et al.*, 1999), as well as acting via TrkB receptors in a manner very similar to that of neurotransmitters (Canossa *et al.*, 1997; Kafitz *et al.*, 1999). These findings combined with the results presented in this thesis that neurotransmitters can act like neurotrophins, are beginning to question the definition of what constitutes a neurotrophin versus a neurotransmitter, leading to the question: are the two the same?

In summary, this thesis has examined the effects of neurotransmitters which signal through GPCRs in sympathetic neurons, examining both the short-term and the long term effects on ionic conductances. The primary function of the nervous system is the transfer of information along a neuron via electrical means and between neurons via chemical means. Determining the mechanism of action of neurotransmitters in the sympathetic nervous system is important to understanding the functioning of this system and is relevant to the development of new treatments for a variety of disorders relating to excessive autonomic signaling. Understanding the relationship between chemical transmitter and electrical properties of neurons is fundamental to understanding the functioning of the nervous system as a whole.

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